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BIOCHEMICAL STUDIES OF CHILLING INJURY IN SWEETPOTATOES[1]

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Many plants indigenous to subtropical and tropical regions suffer physiological injury when subjected to low (1 to 10° C) but non-freezing temperatures (6, 14). The injury, manifested by discoloration, susceptibility to decay, and failure to ripen, is generally progressive with time indicating a gradual degeneration of physiological processes. There is also some evidence (7, 16) that low temperature has an immediate effect on cellular metabolism in sensitive tissues. Sachs (16) reported that some plant cells subjected to low temperatures (11° C) showed cessation of cytoplasmic streaming. Lewis (7) recently confirmed these observations with petiole trichomes of tomato (*Lycopersicon esculentum*) and of sweetpotato (*Ipomoea batatas*) and with other species sensitive to low temperatures. These cells showed cessation of protoplasmic streaming when subjected to 10° C for one or two minutes. In contrast, trichome cells of tissues not sensitive to low temperatures, such as those of radish (*Raphanus sativus*) and carrot (*Daucus carota*), showed protoplasmic streaming even at 0° C.

While the effects of chilling injury have been described adequately in many sensitive tissues (6, 14) there have been few metabolic and physiological studies of this phenomenon. It therefore seemed desirable to investigate biochemical aspects concerned with chilling injury. The emphasis in such a study should be to detect changes occurring at the cellular and subcellular level during exposure of sensitive tissues to low temperatures.

We recently studied cytoplasmic particles from sweetpotato roots (a tissue sensitive to low temperature) and had established a system for assaying their mitochondria (8, 9). It was therefore possible to investigate the effect of chilling on mitochondria, the organelles intimately concerned with energy metabolism in the cell. Our purpose was to compare mitochondrial activity from chilled and non-chilled tissues and also to study some metabolic differences between tissue slices from chilled and non-chilled tissues. It is hoped that this approach will yield clues to the mechanisms concerned in chilling injury.

MATERIALS AND METHODS

Porto Rico sweetpotato roots (*Ipomoea batatas* L. (Lam.)) grown on the Plant Industry Station farm at Beltsville, Maryland, were used in these experiments. The roots were harvested early to insure that no chilling occurred in the field. Immediately after harvest, the roots were held for 10 days at 29° C and approximately 95 % relative humidity to promote healing of surface injuries. After this curing period the sweetpotatoes were divided into two lots. One lot was transferred to a constant temperature room held at 15° C, a recommended storage temperature, and the second lot was transferred to a room held at 7.5° C, the chilling temperature used in these experiments. Both rooms were equipped with humidistats set to maintain a relative humidity of 80 to 85 %. The roots were sampled after curing and at predetermined intervals during storage at the two temperatures. The chilled and non-chilled roots were compared with respect to ion leakage from tissue slices and with respect to oxidation and phosphorylation exhibited by isolated mitochondria.

The leakage from tissue slices was determined by conductivity measurements of solutions surrounding slices which were approximately 2 mm thick and 1 cm in diameter. Duplicate 10-g samples from chilled and non-chilled roots, previously washed in distilled water for one hour, were placed in 120-ml flasks containing 15 ml of either distilled water or 0.5 M sucrose. The flasks were shaken in a constant temperature bath at 25° C for two hours at a shaking speed of 120 strokes per minute. Conductivity measurements were made with a Serfass Conductivity Bridge using a cell with a constant of 0.1. The tissue was sampled at harvest and at intervals as specified during a 10-week storage period.

In the 2nd year the solutions were frozen and held until the end of the experiment (10 weeks), after which time they were analyzed for inorganic ions. The analyses were made by flame photometry following the procedure of Myers, Dyal and Borland (13).

Preparation of mitochondria, assay of mitochondrial activity, and determination of phosphorus and nitrogen were all carried out as described in previous publications (8, 9) except for minor changes as indicated in the tables of this report.

Most of the experiments were carried out in duplicate in each of two years.

RESULTS

CONDUCTIVITY MEASUREMENTS AND ION LEAKAGE: Conductivity of the solutions (water and 0.5 M su-

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TABLE I

SPECIFIC CONDUCTIVITY (MICROMHOS/CM) OF MEDIA SURROUNDING SLICES OF SWEETPOTATO TISSUE AFTER TWO HOURS SHAKING IN A CONSTANT TEMPERATURE BATH AT 25° C *

PERIOD IN STORAGE	TISSUE IN WATER		TISSUE IN 0.5 M SUCROSE	
	NON-CHILLED	CHILLED	NON-CHILLED	CHILLED
None	2.4	...	1.0	...
3 weeks	2.2	6.5	1.0	1.9
4 weeks	2.8	7.8	1.0	2.9
7 weeks	3.2	10.4	1.3	4.5
9 weeks	2.8	10.2	1.2	6.5
10 weeks	2.8	9.6	1.0	5.0

* Conductance readings were made at 23° C with a Serfass conductivity apparatus using the 1,000 cycle per second bridge and a conductivity cell with a constant of 0.1. The data for the two years did not vary more than 10 % from the means.

crose) surrounding the tissue slices in the vessels was determined after two hours of shaking. Table I presents the average of the results obtained from two years of work. Approximately a 2-fold increase in conductivity was noted in the water around the chilled tissue. This difference was observed after 3 weeks storage of roots at 7.5° C and was more or less sustained throughout the experiments. Conductivity in the sucrose solutions around the chilled tissues showed approximately the same ratio of increase, but the values were considerably lower (table I). These data indicate that low temperature may cause an impairment of cellular permeability resulting in leakage from the cells.

The nature of the ions leaking from the tissues was investigated by flame photometry. The analyses showed only traces of manganese, iron, aluminum, sodium, and copper in samples from chilled and non-chilled tissues. Since none of these ions leaked from the tissues it seems that they have no relation to the chilling effect. However, there was considerable leakage of potassium ions from the chilled tissue slices (fig 1). Apparently the greater conductivity of the medium around chilled tissue was due entirely to leakage of potassium ions. It was surprising to find that more potassium leaked from the tissues in sucrose than in distilled water even though the conductivity of the sucrose solutions was lower than that of the distilled-water solutions (table I, fig 1). This is probably related to the effect of a non-polar molecule like sucrose on conductivity in an aqueous medium.

MITOCHONDRIAL ACTIVITY: Mitochondria isolated from chilled and non-chilled tissues were compared with respect to their oxidative and phosphorylative capacities. In complete assay systems, the oxidative rates of mitochondria from chilled and non-chilled tissue (table II) were approximately the same during the first four to five weeks. The mitochondria from the non-chilled tissue continued to maintain approximately the same level of oxidation throughout the 10-week period of the experiments. However, the mitochondria from the chilled tissue showed a marked decline after the 5th week and at the 10th week there was practically no activity at all.

Phosphorylation, as indicated by P/O ratios (table III), was reduced in the chilled tissue even before there was a reduction in oxidative activity. After the 5th week the decline in the P/O ratio became considerably more pronounced, and after the 8th week phosphorylation was practically eliminated.

These data indicate that if the oxidative pathway in the mitochondria was injured by chilling, the injury was reversible during the 1st four- to five-week period at low temperature. There was some decline in the phosphorylating system during this period, but only after five weeks were both oxidation and phosphorylation seriously degraded. It seems logical to suppose, therefore, that the initial injury caused by low temperature does not seriously affect the mitochondria per se but may affect other cellular components which ultimately have considerable influence on mitochondrial activity.

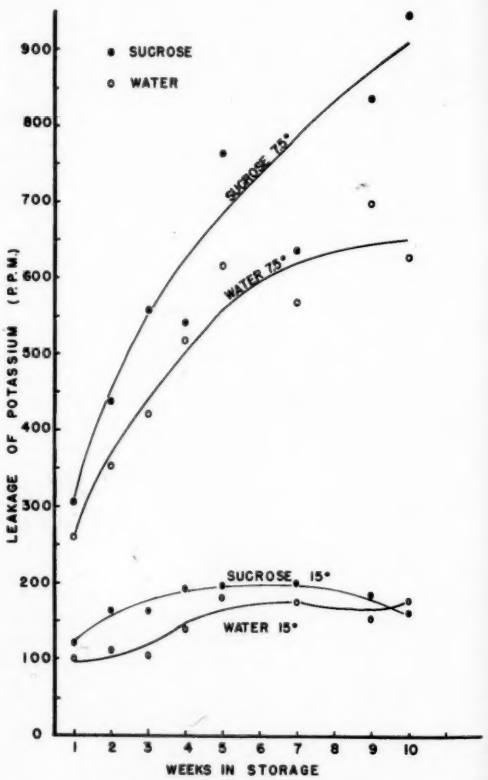

FIG. 1. Leakage of potassium ions from tissue slices of sweetpotato roots stored at a chilling temperature (7.5° C) and at a non-chilling temperature (15° C).

INTER-RELATION OF POLYPHENOLS AND ASCORBIC ACID DURING CHILLING: One of the striking characteristics of chilled sweetpotato roots is the darkening of the tissue on exposure to oxygen. This rapid darkening occurs after a prolonged period at low temperatures and is therefore a manifestation of some of the final degradation reactions caused by low temperature.

During the mitochondrial studies of the badly chilled tissues we observed that the supernatants, obtained by high-speed centrifugation of the homogenates, turned black on standing. The supernatants from tissues that had been exposed to low temperature for shorter periods did not turn black. It therefore seemed that a substrate either accumulated or suddenly appeared as a result of chilling and that it was oxidized to form a dark pigment. Chlorogenic acid was a likely suspect since it is a polyphenol which can be oxidized to melanin-like pigments and has been identified in the sweetpotato root (15).

Comparisons of the chlorogenic acid content in the chilled and non-chilled tissues were made by extracting 100 g of sweetpotato roots (previously held for 12 weeks at 7.5° C) in a Waring blendor at top speed for five minutes with 150 ml of 95 % ethanol. The filtered extract was brought to 250 ml with 50 % ethanol and a 1-ml aliquot was diluted to 250 ml with distilled water. The absorption spectra of these diluted samples were read in the Beckman DU spectrophotometer from 260 to 380 mμ. The ultraviolet absorption spectra of these extracts, compared with the spectrum of authentic chlorogenic acid, are shown in figure 2. These data clearly demonstrate a considerable increase of chlorogenic acid, or related polyphenols which have similar absorption spectra, in the chilled tissues.

The original alcoholic extracts from the chilled and non-chilled tissues were spotted on Whatman no. 1 paper and co-chromatographed with authentic chlorogenic acid in butanol : acetic acid : water (4 : 1 : 5). When the paper was viewed under ultraviolet light, four fluorescent spots were visible from both extracts, indicating that the chilled and the non-chilled tissues contained essentially the same components capable of absorbing ultraviolet. However, the spots derived from the chilled tissues were considerably larger and

TABLE II

OXYGEN UPTAKE BY MITOCHONDRIA FROM CHILLED AND NON-CHILLED SWEETPOTATOES EXPRESSED AS Q_{O_2} (N) *

PERIOD IN STORAGE	1ST YEAR **		2ND YEAR **	
	NON-CHILLED TISSUE	CHILLED TISSUE	NON-CHILLED TISSUE	CHILLED TISSUE
None	494	494	550	550
3 weeks	523	518	1138	1180
4 weeks	345	353	717	851
5 weeks	500	500	745	342
7 weeks	458	347	740	532
8 weeks	530	207	778	218
9 weeks	552	142	920	219
10 weeks	398	11	937	64

* The assay system consisted of 0.01 M α-ketoglutarate, 0.001 M adenosinetriphosphate, 0.02 M glucose, 1 mg hexokinase, 3.3×10^{-4} M diphospho pyridine nucleotide, 6.6×10^{-5} M cocarboxylase, 6×10^{-3} M Mg, 0.02 M PO_4, and 0.5 ml mitochondria containing approximately 0.3 to 0.5 mg of nitrogen.

** The homogenizing medium for isolating the mitochondria differed in the two years. In the 1st year the medium contained 0.25 M sucrose, and 0.01 M EDTA and yielded mitochondria containing approximately 0.5 mg of N per aliquot. In the 2nd year the medium contained 0.25 M sucrose, 0.1 M phosphate buffer pH 7.0, and 0.01 M EDTA and yielded mitochondria containing approximately 0.3 mg of N per aliquot. This is believed to be responsible for the higher Q_{O_2} (N) values during the 2nd year.

TABLE III

AVERAGE P/O RATIOS FOR OXIDATIVE SYSTEMS USING MITOCHONDRIA ISOLATED FROM CHILLED AND NON-CHILLED SWEETPOTATO TISSUE *

PERIOD IN STORAGE	NON-CHILLED TISSUE	CHILLED TISSUE
None	1.0	1.0
3 weeks	1.2	0.9
4 weeks	1.8	1.1
5 weeks	1.9	1.0
7 weeks	1.3	0.6
8 weeks	1.2	0.6
9 weeks	1.9	0.1
10 weeks	1.4	0.1

* The data are averages for two years; same assay system as in table II.

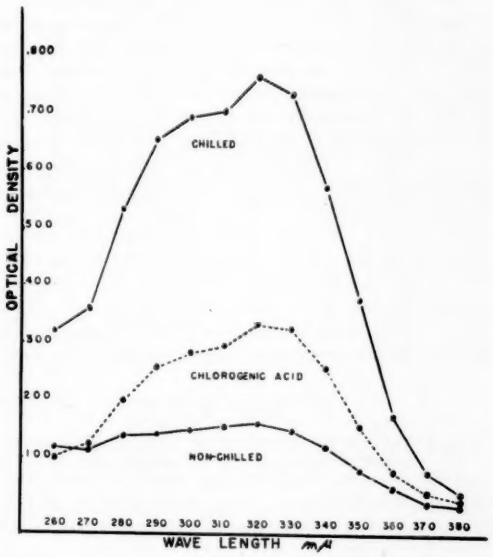

FIG. 2. Ultraviolet absorption spectra of extracts from chilled and non-chilled sweetpotato roots and of authentic chlorogenic acid (2×10^{-5} M).

TABLE IV

R_f VALUES OF SPOTS OBTAINED ON PAPER AFTER CHROMATOGRAPHING EXTRACTS FROM CHILLED AND NON-CHILLED SWEETPOTATO TISSUES

SPOT NUMBER	NON-CHILLED TISSUE		CHILLED TISSUE	
	R_f VALUE	BLUE-GREEN SPOT PRESENT AFTER FUMING WITH NH_3	R_f VALUE	BLUE-GREEN SPOT PRESENT AFTER FUMING WITH NH_3
1	.43	–	.43	–
2	.58	+	.58	+
3	.75	+	.75	+
4	.88	+	.88	+
Authentic chlorgenic acid	.75	+	.75	+

more dense in all cases, indicating higher concentrations. This is in agreement with the ultraviolet absorption spectra. One of the spots derived from both the chilled and the non-chilled tissue coincided with authentic chlorogenic acid. The R_f values for the four spots are shown in table IV.

The data in figure 2 and the density of the spots on the chromatograms indicate a considerable increase in chlorogenic acid in the chilled tissues. There also appears to be a like increase of the other substances (spots 1, 2, and 4) appearing on the paper. Spots 2 and 4 may be neochlorogenic acid and *iso*-chlorogenic acid, respectively, since the R_f values appear to be approximately the same as those reported for these substances (4) and the ultraviolet absorption spectra of these three chlorogenic acids are identical (19).

Ascorbic acid has been shown to reduce quinones to phenols (11) and also has been reported to have an inhibitory effect on the polyphenolase enzyme complex (1). Craft and Heinze (3) found no relation of ascorbic acid to chilling of tomatoes while Miller and Heilman (12) found that ascorbic acid declined when the pineapple (*Ananas comosus*) was subjected to low temperatures. It was, therefore, interesting to compare the ascorbic acid in chilled and non-chilled sweetpotatoes. Ascorbic acid concentrations were determined by the method of Loeffler and Ponting (10). The result of such a study (table V) showed that a marked decline in ascorbic acid occurred in chilled tissues whereas the ascorbic acid content of the non-chilled tissue remained approximately constant.

TABLE V

CONCENTRATION OF ASCORBIC ACID DURING STORAGE OF SWEETPOTATOES AT NON-CHILLING AND CHILLING TEMPERATURES

PERIOD IN STORAGE	NON-CHILLED TISSUE	CHILLED TISSUE
10 weeks	22.7	9.2
11 weeks	21.1	5.3
12 weeks	20.9	7.6
13 weeks	20.1	8.3

These data were taken after a prolonged period at the chilling temperature (7.5° C), and they indicate the general course of the final degradative processes. It appears that in chilled sweetpotato tissue there may be a decrease in ascorbic acid accompanying the increase in polyphenols.

DISCUSSION

After observing increased leakage in tissue slices from roots held three weeks at 7.5° C, it was surprising to find normal oxidative activity in the mitochondria isolated from the roots stored for more than four weeks at 7.5° C. These data may be explained by assuming that the mitochondria per se are not affected by low temperature, but that the nature and the concentration of the cofactors, which control the mitochondrial activity in vivo, are affected. Since in our assay system all the known cofactors were liberally supplied, these experiments offer no test of limitation of cofactors for mitochondrial activity in vivo. For example, potassium which we know leaked from chilled tissues, was adequately supplied with the phosphate buffer. In an alternate explanation, one may assume that the degree of injury to the mitochondria caused by 7.5° C for three or four weeks is considerably less than that due to the normal isolating procedure. Therefore, no effect on the mitochondria is observed after chilling the tissue for a short period at 7.5° C.

We may consider five to six weeks at the chilling temperature (7.5° C) as the "breaking point" in chilled sweetpotatoes. It is after this period at low temperatures that the mitochondria are severely injured, and their activity cannot be restored by the addition of cofactors. Also, at about this time chlorogenic acid begins to accumulate and ascorbic acid starts to decline. The data for these last observations are not reported in this paper, but we have observed this in a later study to be published elsewhere.

The loss of potassium from the chilled cells may be one of the factors concerned in the deterioration of oxidative phosphorylation after five or six weeks of chilling the roots. Lardy (5) has suggested that potassium ions may be involved in uncoupling oxidative phosphorylation. However, several observers (2, 17) reported that potassium ions are released from cells as a general effect of exposure to radiation. The release of potassium from chilled cells may thus be a non-specific effect of cellular injury.

Shichi and Uritani (18) suggested that a gradual coagulation of the lipid components of the sweetpotato occurs at low temperatures and damage to cellular structures eventually results. Support for this hypothesis may be derived from the long term chilling requirement in sweetpotatoes as evidenced by the five to six week "breaking point" (at 7.5° C) previously discussed. On the other hand the rapid leakage of potassium from chilled cells and the observations by Lewis (7) of the rapid cessation of cyclosis at low temperatures militates against this hypothesis.

More work is needed to explore additional physiological and biochemical aspects of low temperature injury.

Summary

Chilled and non-chilled sweetpotato roots were compared with respect to leakage from tissues, and mitochondrial activity during 10 weeks of storage at 15 and 7.5° C (the chilling temperature).

There was approximately five times as much leakage from the chilled tissue slices as from the non-chilled tissue slices. Almost all the leakage consisted of potassium ions.

During the 1st four weeks, the mitochondria from chilled and non-chilled tissues, when assayed in complete systems at 25° C, showed approximately equivalent rates of oxidation and phosphorylation. However, after the 5th week there was a rapid decline in oxidative and phosphorylative activity in the chilled tissues and at the 10th week the mitochondria from the chilled tissues were practically inactive.

In the late stages of degeneration there was a build up of chlorogenic acid (and similar polyphenols) and a decline of ascorbic acid in the chilled tissue.

We gratefully acknowledge the assistance of A. Specht of the Plant Industry Station, who determined the potassium concentrations in the sucrose and water solutions surrounding the tissue slices.

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AUXIN ACTIVITY OF SOME INDOLE DERIVATIVES[1,2]

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During the past 20 years, in numerous laboratories, very large numbers of compounds have been synthesized and tested for their activity as auxins or as modifiers of auxin action. The majority of these have been acids of the aryloxy series. Curiously enough, although indoleacetic acid (IAA) is the most widely occurring natural auxin, very few of the compounds tested have been indole derivatives. Kostermans in 1934 tested a number of indole derivatives, but only in the Avena curvature test with agar blocks, where the ability of the substance to be transported largely controls its activity. More recently,

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using Avena coleoptile sections in solution, Muir and co-workers (14, 15) tested 4-chloroindoleacetic acid and found it to have 80 % (15) or 140 % (14) of the activity of IAA; 2-methyl-IAA was found to have 1.5 % (14) or 45 % (15) of the activity of IAA, and introduction of two chlorine atoms into the ring did not raise this. Hansen (7) found 6-chloro-IAA more active in inhibiting root growth and root cell elongation than IAA. Hansen et al (8) and Fawcett et al (5) have tested indoleisobutyric acid (see below), and van de Westeringh (28) has shown that 3-indolemethylsulfonic and phosphonic acids and 3-indolemethyltetrazole have some activity, though unfortunately his data were not converted to relative activity or any other uniform quantitative measure. With the exception of these few cases, no systematic testing of indole derivatives has been carried out.

The present paper is a beginning at making up this deficiency; it reports tests which have been carried out at various times over a period of 8 years on 23 synthetic indole derivatives or closely related compounds. In a number of cases the results are of special interest for their bearing on current hypotheses or controversies. They illustrate again some of the striking specificities in the relation between structure and auxin activity.

Materials and Methods

Three bioassays have been employed: straight growth of 10-mm subapical sections of 72-hour Segrehaver Avena coleoptiles, straight growth of 20-mm sections from the immediately subapical part of the 3rd internode of etiolated 7-day-old Alaska pea stems grown in the presence of occasional weak red light, and curvature of longitudinally slit sections of the same pea stems.

Details of the methods have previously been given (21, 24, 25). Since the slit pea stem curvature is, on the whole, the least selective (25) it was generally used for the 1st screening. The Avena tests were carried out with 10 to 12 10-mm sections floating on 4 ml of solution in a 4-cm Petri dish. The solution contained 2 % sucrose, M/200 or M/1000 KCl or, in later work, potassium phosphate M/400 brought to pH 4.5 with HCl. $MnCl_2$ 10^{-4} M was added in a few of the earliest tests, and after 1954 (21) 3×10^{-5} M $CoCl_2$ was used.

The pea tests were usually carried out in 20 ml of solution in a Petri dish, without additions other than the test substance, but in a few instances M/100 KCl or buffer was added. Although the addition of $CoCl_2$ plus sucrose to pea sections increases the growth (sucrose alone does not do this under our conditions) the increment caused by addition of an auxin is not proportionately increased.

The elongation or curvature was routinely measured after 22 hours of gentle rotary shaking in darkness unless otherwise indicated. Some variation in the growth of controls between different experiments is due largely to the various small changes in technique which have been made during the 8-year period. Changes of a few tenths of a degree in the temperature of the dark room are also responsible for appreciable variations in growth rate.

Indoleacetic acid (IAA) in several concentrations was included in all assays, and wherever the activity of a compound was appreciable, it is expressed as percent of the activity of IAA, by interpolation on the IAA curve, as exemplified in tables III and IV. To simplify the formulae, the indole radical is presented as *Ind.* wherever possible.

The compounds tested and their sources were as follows: Indole, Eimer and Amend Co.; 5-methylindole, Sigma Chem. Co.; 6-methoxyindole, synthesized by Fritz E. Bader of Harvard; skatole, Eastman Kodak Co.; indole-3-glycolic acid, sample of Na salt supplied by Professor Marvin D. Armstrong and synthesized by K. N. F. Shaw of the University of Utah; indole-3-glyoxylic ethyl ester, melting point 188 to 189° C (higher than in the literature), synthesized by Bruce B. Stowe of Harvard; N-acetylindoxyl, samples from Bios Ltd. and Delta Chemical Co.; five oxindole derivatives, synthesized by J. B. Hendrickson of Harvard; 3-methyldioxindole, melting point 167 to 168° C, synthesized by Bruce B. Stowe of Harvard; α-indole-3-isobutyric acid, supplied by Professor H. Burström (3) and synthesized by G. N. A. Jönsson of the Royal Institute of Technology, Stockholm; 5- and 7-hydroxyindoleacetic acids, synthesized by A. Ek and B. Witkop (4); indole-3-lactic acid, melting point (sharp) 100° C, isolated from a culture of *Oidium lactis* on a tryptophane medium by Bruce B. Stowe of Harvard; indolemethylenemalonic acid and three imidazole derivatives, synthesized by Bernhard Witkop of the National Institutes of Health; indene-3-propionic acid, synthesized by Professor G.

Table I

Action of Indole Alone and in Combination with 2-Carbon Compounds on Growth of Avena Coleoptile Sections *

Control **	Plus indole 1×10^{-4} M	Plus addition	3×10^{-3} M	10^{-3} M	3×10^{-4} M	10^{-4} M
38.3	45.2	Glycolate	37.9	41.5	45.4	... ***
38.7	40.3	Chloracetate	6.4	17.2	32.0	34.6
38.7	40.3	Glycine	35.2	40.6	38.8	38.2

* All figures are elongation after 22 hours as percent of initial length.
** Sucrose 2 %, KH_2PO_4 pH 5.5 M/200, $CoCl_2$ 3×10^{-5} M.
*** No promotion in another experiment.

TABLE II

ACTION OF INDOLE ALONE AND IN COMBINATION WITH D-RIBOSE ON GROWTH OF AVENA COLEOPTILE SECTIONS *

CONCN OF INDOLE	CONCN OF RIBOSE		
	0	10^{-4} M	10^{-3} M
0	34.3 **	35.0	38.2
3×10^{-6} M	40.0	37.0	40.0
1×10^{-4} M	41.6	...	...
3×10^{-4} M	50.9	50.1	47.4

* All figures are elongation after 22 hrs as percent of initial length. Mean of 2 complete experiments.
** Control: sucrose 2%, KH_2PO_4 M/200, pH 5.0, no $CoCl_2$.

R. Clemo of King's College, Newcastle-on-Tyne; indole-4-acetic acid, melting point 205° C, synthesized by H. Plieninger and K. Suhr (15a) and supplied by W. A. Andreae of Science Service Laboratory, London, Ontario; 7-azaindole-3-acetic acid, melting point 213 to 214° C, synthesized by M. M. and B. L. Robison (17) of Amherst College, Massachusetts.

The reference sample of IAA was from Hoffmann La Roche, Basel, Switzerland.

1. INDOLE: On a number of occasions a small but real growth-promoting effect of pure indole has been observed, particularly on Avena sections. For instance, in one test, controls in sucrose-KCl grew 45% while sections in 3×10^{-4} M indole grew 66%, an increase of more than one third. As a mean of four other experiments, controls grew 36.5 ± 1.7% and sections in 3×10^{-4} M indole grew 48.7 ± 3.2%, again an increase of one third. Other data appear in tables I and II. The optimum concentration of indole was found to be 3×10^{-4} M in all experiments.

Since indole is unlikely to be an auxin itself, it seemed reasonable to suppose that it might be acting as substrate for the in vivo synthesis of indoleacetic acid. As likely participants in such an in vivo reaction, three substituted acetic acids, namely glycolic acid, chloracetic acid and glycine were tested in combination with indole. However, table I offers no evidence to support the occurrence of a reaction of this type in Avena. The participation of D-ribose in such a synthesis (cf. the synthesis of indole in bacteria from diphospho-ribose and anthranilate (31)) is made improbable by table II.

It must be concluded that if indole is in fact converted by the plant to an auxin, then the material furnishing the side-chain is not any one of the substances tested. Greenberg et al (6) have demonstrated a non-enzymatic condensation of indole with glyoxylic acid, but their product was considered to be indole-3-glycolic acid, and this has only very low activity on Avena, as shown below.

The possibility remains that indole reacts in some way with the endogenous IAA, either protecting it from destruction or synergizing with it. Marked synergism between indole and IAA in root formation was observed by van Raalte (29), but in his experiments indole by itself was without effect.

2. 5-METHYLINDOLE AND 6-METHOXYINDOLE: Both of these compounds are inactive. At 10^{-4} M and above they were found to cause marked browning of Avena sections, especially in presence of $CoCl_2$.

3. SKATOLE: This compound bears the same relation to IAA as 2,4-dichloroanisole does to 2,4-D, namely it is the carboxyl-free analogue. In 1949 Bonner reported (2) that 2,4-dichloroanisole acted as an auxin antagonist. The deduction was made that the interaction between dichloroanisole and IAA, at IAA concentrations below 1 mg per liter, was competitive. However, the published data (table 11 of ref. (2)) show that there was an increase in the percentage of inhibition with increasing concentration of IAA and therefore do not support this claim. Later, Audus and Shipton (1) could find no evidence of competition, but McRae and Bonner (13) supplied more extensive data which did show competition both with IAA and with 2,4-D. However, since it is IAA, not 2,4-D, which is the natural auxin, it seemed much more worth-while to see whether the carboxyl-free analogue of IAA, namely skatole, had inhibiting action, and if so, whether it was competitive with IAA.

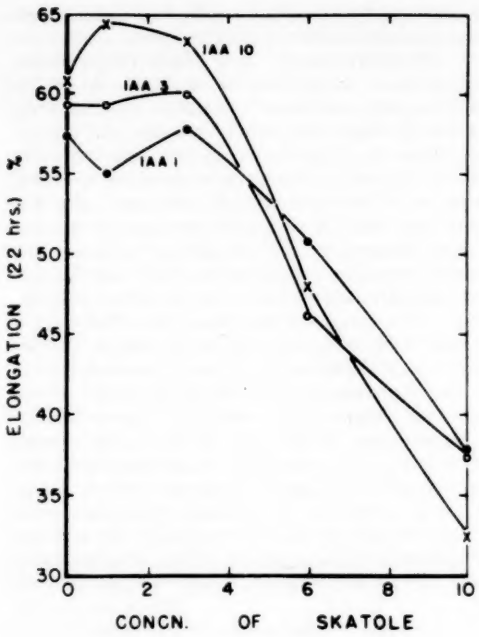
In preliminary experiments (which were reported in 1953 at the Auxin Conference held at Lund, Sweden) skatole was found to inhibit growth in all the test methods, the effective concentration range lying between 10^{-3} and 10^{-4} M. More extensive results with the straight growth of pea stem sections are summarized in figure 1 and data with pea curvature are shown in table III. The straight growth tests show that when the IAA concentration is raised from 1 to 10 mg per liter, neither the percentage of inhibition nor the absolute change in the elongation of the sections is decreased. In fact, the reverse is the case; if, for instance, we take skatole at 6 mg per liter, and calculate the decrease in growth as percent of the control, we find that when IAA is at 1 mg per liter the decreases are 11.5 and 10.3% in two tests,

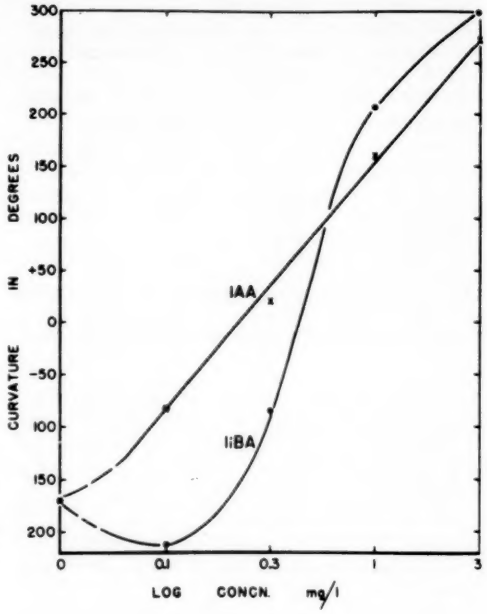
TABLE III

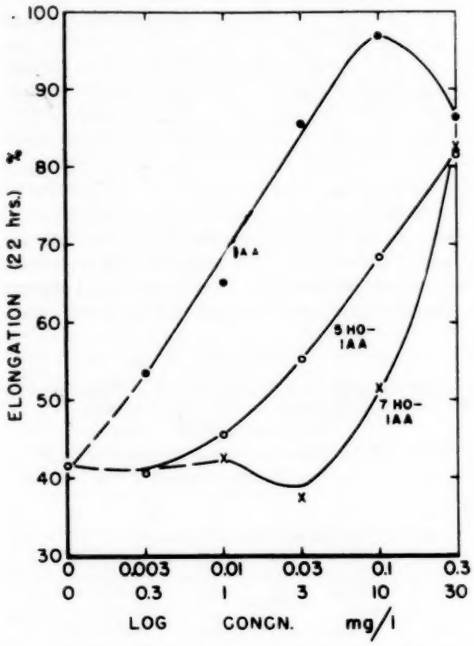
CURVATURE OF SLIT PEA STEMS IN A SERIES OF CONCENTRATIONS OF IAA AND SKATOLE. TEST OF 10/18/49

CONCN OF IAA $\times 10^{-6}$ M	CONCN OF SKATOLE $\times 10^{-4}$ M					
	0	1	3	Δ *	10	Δ *
0	−156	−181	−168		−196	
				− 12		− 40
6	+ 90	+157	+221		−111	
				+131		−201
18	+194	+166	+289		−158	
				+ 95		−352
60	+401	...	+218		− 62	
				−183		−463
180	+447	...	+368		−124	
				− 79		−570

* Change due to skatole.







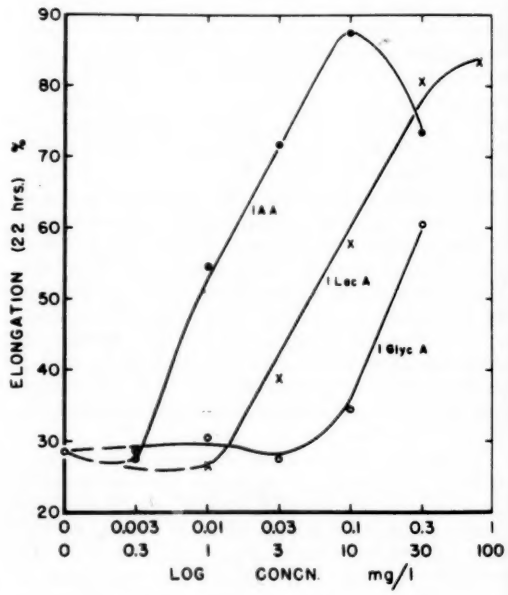


FIG. 1 (*top left*). Inhibiting action of skatole on straight growth of Pisum stem sections at three levels of IAA. (Expt. of 26/10/49). A 2nd experiment gave closely similar results.

FIG. 2 (*top right*). Curvature in the pea test of indole-3-isobutyric acid (IiBA) and IAA. All points from a single experiment (of 13/2/57).

while when IAA is at 10 mg per liter the decreases are 20.8 and 16.4 %. Corresponding figures can be calculated from the data at a skatole concentration of 10 mg per liter. (There is evidence of slight but real growth promotion when the concentration of IAA is high and that of skatole is low.) These data give no indication of any competition between skatole and IAA. Even when the lengths were measured at 6 hours, which is within the period of linear elongation rate for pea stem sections, the inhibition was not decreased in the least by the presence of IAA at 1 or 10 mg per liter. On the whole it tended to be greater rather than less. A Burk-Lineweaver plot of the data of figure 1 and of other similar experiments shows no convergence whatever of the lines for different skatole concentrations.

The curvature tests (table III) lead to the same conclusion. Again there is some promotion or synergism at low skatole concentrations (1 and 3×10^{-4} M). Indeed, it is clearer here than with the straight growth tests. At 3×10^{-3} M, skatole was toxic at all levels of IAA. Readings with the curvature test were not made during the period when the curvature is developing linearly, but nevertheless the results are very clear; high levels of IAA show no tendency to overcome the inhibition caused by skatole. Indeed, the decrease in curvature caused by a given level of skatole is actually greater at high than at low IAA concentration; in other words the behavior is the opposite of that to be expected for competition.

It is concluded that skatole is a growth inhibitor, but that the inhibition is not competitive with auxin, at least in pea stems. The inhibition tends rather to resemble that caused by arsenite, which is greatest at high auxin concentration (21).

4. Indole-3-glycolic Acid: As mentioned above, this substance, *Ind*.CHOHCOOH, has only slight, though real, activity on Avena (see table IV and fig 4). The two tests summarized in table IV yielded relative activities (IAA = 100) of 0.5 and 0.3 % respectively. Minimal or threshold activity is observable at 10 mg per liter. The greater growth in experiment II is largely due to the presence of cobalt, at its optimal concentration, in this experiment.

On Pisum straight growth the relative activity of indole-3-glycolic acid was somewhat lower than on Avena, being about 0.2 %. These data are consistent with the figures given by Greenberg et al (6), from which a relative activity of less than 0.2 % can be calculated. In Pisum curvature, however, a figure of about 2 % was found. It must be concluded that the activity of this compound in all assays, though real, is very low.

5. Indole-3-glyoxylic Ester: Tests with this substance, *Ind*.COCOO · C_2H_5, were inconclusive.

Table IV

Relative Activity of Indoleglycolic Acid on Avena Coleoptile Sections

Substance	Concn mg/l	Elongation in percent	
		I	II
Control solution *	...	51.4	59.8
Indoleacetic acid	0.1	...	89.9
	0.3	63.8	118.6
	1.0	75.3	101.5
Indoleglycolic acid	0.3	48.6	59.7
	1	47.7	61.4
	3	49.3	58.3
	10	49.5	65.2 **
	30	57.8	91.6
	90	69.6	84.0
Equivalence		IAA 0.3 = IGA 60	IAA 0.1 = IGA 30
∴ Relative activity		0.5 %	0.3 %

* Sucrose 2 %, KH_2PO_4 M/200; expt. I without cobalt; expt. II with $CoCl_2$ 3×10^{-5} M.

** Mean of three.

Whether used directly as the ester, or after a few minutes of hydrolysis with warm dilute NaOH, the activity was seldom proportional to concentration. In one Pisum curvature test a relative activity of 0.7 % was indicated, but in another it was zero. High concentrations slightly inhibited Avena growth. A relative activity of a few tenths of 1 %, with slight toxicity at 30 mg per liter, would be consistent with the results. The sample was not large enough for extensive purification, and the danger that it might be decomposing in the solution made a further detailed study unwarranted.

6. N-Acetylindoxyl: This substance has been reported active on Avena (18) and indeed the sample obtained from Bios Ltd. showed slight but irregular traces of activity on Avena. At 10 and 30 mg per liter, however, growth was inhibited about 10 % (as with indole-glyoxylic ester). On the other hand, the sample from the Delta Chemical Co. showed no activity whatever, nor did either sample show any activity on Pisum, either for straight growth or for curvature. It is concluded that N-acetylindoxyl has no auxin activity.

7. Oxindole Derivatives: Since it appears that the product of enzymic oxidation of IAA is an oxindole derivative (16, 19), a number of oxindole compounds were examined. None of the following showed activity on pea curvature in concentrations up to 30 mg per liter, or about 2×10^{-4} M; oxindole-3-acetonitrile, oxindole-3-pyruvic oxime, oxindolene-3-ace-

Fig. 3 (*lower left*). Straight growth of Avena coleoptile sections in 5-hydroxy and 7-hydroxy-indoleacetic acids, compared with IAA. The scale for the two hydroxy acids is the lower one, i.e., their concentrations are 100 times those of IAA.

Fig. 4 (*lower right*). Straight growth of Avena coleoptile sections in indole-3-lactic (ILacA) and indole-3-glycolic (IGlycA) acids, compared with IAA. The scale for ILacA and IGlycA is the lower one.

tonitrile, oxindolene-3-pyruvic acid and oxindolone (3-dehydrodioxindole).

Special attention was paid to 3-methyldioxindole, since this compound has many properties in common with the IAA oxidation product (19). Tested on Avena up to 30 mg per liter in the presence of IAA concentrations from 0 to 5 mg per liter, it showed neither auxin activity, synergism nor true inhibitory effect. Pea curvature tests showed the same lack of all three activities.

8. Indole-3-isobutyric Acid: Burström (3) and Hansen (7) have shown indole-3-isobutyric acid (IiBA), or *Ind*.$C(CH_3)_2COOH$, to be a powerful "root auxin," promoting the elongation of wheat roots by about 50 %, and opposing the growth-inhibiting action of IAA. It would be expected, therefore, to act as an auxin antagonist in the present tests. The reverse is the case. On Avena its effect is weak, but the slight activity is growth-promoting—about 0.3 % of IAA; however, the very slow increase in growth with increase in IiBA concentration makes precise comparison impossible. The linear relation and gradual slope are shown in figure 5, which confirms the curve given by Hansen et al (8). Two pea straight growth tests likewise showed real growth promotion, with relative activities of 1.5 % and 2 % respectively.

It is the activity in the pea curvature test that is remarkable. Here IiBA actually produces larger curvatures, at high concentrations, than does IAA (fig 2). This behavior, coupled with the low activity and unusual concentration-dependence in the Avena section test, makes it impossible to ascribe the activity of IiBA to traces of indole-*n*-butyric acid as an impurity.

In the pea curvature test, the difference in slope of the curves for IAA and IiBA (see fig 2) makes it difficult to assess the relative activity of IiBA. Using as criterion the concentration needed to reduce the outward curvature by 100° (25), the relative activity would be 33 %; however, using the concentration needed to produce 100° inward curvature, it would be over 100 %, i.e., IiBA would be more active than IAA. This high activity in differential growth compared with the much lower values for straight growth indicates that IiBA is probably transported from cell to cell only with difficulty. It is of interest, also, that in the similar curvature test with slit Avena coleoptiles (24), IiBA is significantly more active than on Avena straight growth, the relative activity approaching 25 %. Thus it is largely the curvature method per se which is responsible for the high relative activity.

At no concentration did IiBA inhibit growth, in the absence of added IAA. However, the increased growth of Avena due to IAA was clearly inhibited by IiBA. A concentration 10 times that of the IAA reduced the growth increment by about 10 %. At lower ratios, and especially at low absolute concentrations of IAA, there is some evidence of synergism, especially in pea straight growth.

In contrast to its action on roots, IiBA is thus clearly an auxin for shoots. A similar conclusion has been drawn by Fawcett et al (5).

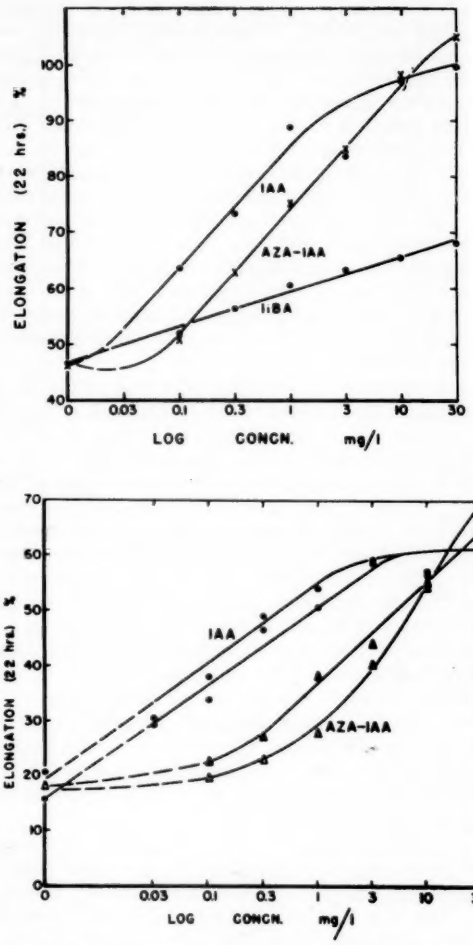


Fig. 5 (*above*). Straight growth of Avena coleoptile sections in 7-azaindoleacetic acid (Aza-IAA) and indole-3-isobutyric acid (IiBA). Tests of 16/7/56 (Aza) and mean of 25/11/57 and 2/12/57 respectively (IiBA). All on same scale.

Fig. 6 (*below*). Two pea stem straight growth bioassays of 7-aza-IAA, each with its reference curve for IAA (31/10/56 and 1/11/56).

9. 5-Hydroxy- and 7-Hydroxyindoleacetic Acids: The former of these two acids has recently come into prominence as the product of the physiological oxidation of 5-hydroxytryptamine or serotonin. Quantities up to 400 mg per day may be excreted in human urine in certain pathological conditions (26), and the feeding of tryptophane also leads to its excretion (27). Furthermore, 5-hydroxy-IAA occurs in plants, having been found in seeds of *Piptedenia peregrina* (cited in (27)). It is also of importance

TABLE V

CURVATURE OF SLIT PEA STEMS CAUSED BY 5- AND 7-HYDROXYINDOLEACETIC ACIDS

CONCN, MG PER LITER	CURVATURE IN IAA (MEAN OF 3)	CURVATURE IN 5-HO-IAA (MEAN OF 2)	CURVATURE IN 7-HO-IAA
100	...	+201	+194
30	...	+172	− 34
10	+395	+ 3	− 42
3	+228	−118	−342
1	+104	−221	−234
0.3	+ 2	−175	−221
0.1	− 83	...	...
H_2O	−190	−190	−198
Relative activity		4 %	1.6 %

that both these acids can be attacked by the very specific indoleacetic acid oxidizing enzyme from *Omphalia flava*, albeit the action is slow and the products are not the same as those formed from IAA (16).

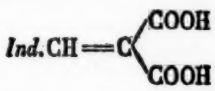
On Avena coleoptile sections both compounds show weak activity. In figure 3 the scale for the hydroxy acids is 100 times that for IAA; the relative activity of 5-hydroxy-IAA averages 0.12 % of that of IAA, while 7-hydroxy-IAA appears less active, perhaps 0.06 %. A feature of these tests is the marked deposit of purple breakdown products at the cut surfaces of the sections. This is particularly marked with 7-hydroxy-IAA. This suggests that if inactivation at the cut surface could be reduced, without inhibiting growth, the relative activity might well be higher.

Pea curvature assays are shown in table V. The small sample available of the 7-hydroxy acid did not permit much repetition of tests, but it is clear that both substances possess real, though low, activity, and that the 5-hydroxy acid is about twice as active as the 7-hydroxy acid. The relative activities of 4 % and 1.6 % represent a slight downward revision of the figures cited in (4). On pea straight growth, both substances were virtually inactive up to 30 mg per liter (1.6×10^{-4} M).

10. INDOLELACTIC ACID: The very great lowering of activity occasioned by introduction of a hydroxyl group in the two acids just mentioned lent special significance to a study of indole-3-lactic acid (ILacA), or *Ind*.$CH_2CHOHCOOH$. This compound has recently been identified (12) as among the products formed from tryptophane by *Agrobacterium tumefaciens*. On Avena sections the growth increment caused by ILacA is satisfactorily proportional to concentration, and the slope of the curve is parallel to that of IAA (fig 4). The relative activity is only 0.16 %. On the straight growth of pea stems the activity was higher, averaging 0.4 % in two tests. As usual, a higher value for pea stem curvature, about 1.8 %, was found.

Although this sample of ILacA had the correct melting point (100° C; given in the literature as 100 to 101° C) it was not a synthetic product but was obtained from *Oidium lactis* (*Geotrichum candidum*); it is difficult, therefore, to rule out the possibility of its containing a minute trace of IAA. Careful chromatography of quantities of 100 μg each, in several different solvents, did not reveal any sign of IAA, so that the amount of IAA present, if any, is probably less than 0.2 %. In any case, since the relative activity of ILacA is significantly different in different tests, and especially since in the pea curvature test it approaches 2 %, the whole of its activity cannot be ascribed to contaminating IAA. However, the activities quoted may perhaps be regarded as maximal.

11. INDOLEMETHYLENEMALONIC ACID: In the chlorophenoxyacetic series it has been shown (10, 11; see discussion in (20)) that the introduction of a 2nd carboxyl group almost completely destroys activity in the pea test. The availability of a small sample of indole-3-methylenemalonic acid,



(used as a synthetic intermediate), made possible another test of this rule. Both on pea curvature and pea straight growth it was found virtually inactive. The relative activity on pea curvature, if real, was clearly less than 0.2 %.

12. IMIDAZOLE DERIVATIVES: The point was made some years ago (20) that thus far no synthetic compounds having only a 5-membered ring have been found active as auxins. Pyrrole-3-acetic, thiopheneacetic, furylacetic and both isomers (Δ_1 and Δ_2) of

TABLE VI

RELATIVE ACTIVITY OF INDOLE-4-ACETIC ACID (I-4-AA) ON AVENA COLEOPTILE SECTIONS, AND TEST FOR INTERACTION WITH INDOLE-3-ACETIC ACID

CONCN, MG PER LITER	ELONGATION IN PERCENT AFTER 22 HOURS				
	I-3-AA	I-4-AA	I-3-AA	I-4-AA	BOTH
Control solution *	32.8		44.3		
0.01	44.2	...	...	...	
0.03	69.0	...	78.3	...	93.3
0.10	70.1	...	...	...	
0.30	71.5	29.9	...	55.5	
1.0	76.9	45.7	...	...	88.1
3.0	72.4	64.8	93.4	...	
10.0	...	69.8	...	...	
30.0	...	71.4	...	...	

Equivalence:

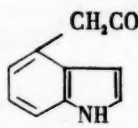
I-3-AA 0.01	= I-4-AA 0.9	or relative activity =	1.1 %
0.03	9		0.3 %
0.1	14		0.7 %
0.3	30		1.0 %

* Sucrose 2 %, KH_2PO_4 M/200 pH 5.0, $CoCl_2$ 3×10^{-5} M, Penicillin G 2×10^{-6} M.

cyclopenteneacetic acid are all inactive. Yet the same structures when fused with 6-membered rings (i.e., the acetic derivatives of indole, thionaphthene, benzofurane and indene) are highly active, at least on pea curvature. The behavior of the three imidazole derivatives so far tested supports this generalization, and although they are not indole compounds it is appropriate to record them here. Imidazoleacetic acid, imidazoleacrylic acid (or urocanic acid) and imidazoleacetonitrile were all completely inactive in the pea curvature test up to 100 mg per liter. They also had no effect on straight growth.

13. Indene-3-propionic Acid: This compound is also not an indole, and is only of interest here because it is so much less active than its acetic homologue. Indene-3-acetic acid has been shown to have 20 % relative activity in the pea curvature test, though less in other tests (see discussion in ref. (20)). Two pea curvature tests with indene-3-propionic acid gave relative activities of only 3 and 4 %. On pea straight growth it was nearly inactive.

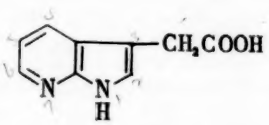
14. Indole-4-acetic Acid: This acid:



was found to have unexpectedly low activity. Representative bioassays, shown in table VI, indicate that on Avena its activity is exactly 1 % of that of IAA. On Pisum curvature (in accord with the usual lesser selectivity of this test) it is about 2 %. In the straight growth of Pisum the curve is steeper than for IAA so that a relative activity can be only roughly estimated. In Avena, however, the slope of the line relating concentration to growth increment closely parallels that for IAA.

In the presence of low concentrations of IAA, indole-4-acetic acid gives no indication of synergism, and in the presence of high concentrations there is little evidence of antagonism (table VI) although some slight interaction is not excluded.

15. 7-Azaindole-3-acetic Acid: Although this compound:



seemed of special interest as a possible auxin antagonist, figure 5 shows that it acts as a powerful auxin on Avena, and indeed, it is easily the most active of the substances described in this paper. Its relative activity, derived from several points on the curve, averages 50 %. In combination with IAA, 7-azaindole-3-acetic acid (7-Aza-IAA), showed no synergism or antagonism.

The two tests on Pisum straight growth (fig 6) show that, at 30 mg per liter, 7-Aza-IAA is at least as active as IAA, while at low concentrations the relative activity falls to 10 %. In pea curvature the activity is essentially equal to that of IAA throughout.

Discussion

The best values for the relative activities are summarized in table VII. The first conclusion to be drawn from the data is that the structural requirements for auxin activity in the indole series are rather demanding. This extends also to the requirements for activity as an antagonist or synergist. It makes a refreshing contrast to the situation with purely aromatic compounds, in which good activity is found in structures ranging all the way from 2,3,6-trichlorobenzoic acid to 1-naphthalenephosphinic acid and 2,4-dichlorophenoxymethyltetrazole (28, 29).

The second conclusion is that the concept of "anti-auxin" or auxin antagonist needs considerable modification. The non-competitive inhibition of pea stem growth and curvature by skatole shows that the suggestion that carboxyl-free analogues should act as anti-auxins is not borne out in this case. The failure of 7-Aza-IAA to act as an antagonist is unexpected in view of the antagonistic activity of other 7-Aza-indoles in other systems (17). The clear growth-promoting activity of indoleisobutyric acid shows that an anti-auxin in one test may be an auxin in another. Similarly, triiodobenzoic acid can be an antagonist in one test, an auxin in another and a synergist in a third (see discussion in (23)). While there certainly can be antagonism in specific tests, there is probably no one substance that can safely be regarded as a

Table VII

Summary of Relative Activities (IAA = 100) in the Three Bioassays

	Avena straight growth	Pisum straight growth	Pisum (slit) curvature
Indole	0.02	...	...
Skatole	Inhib	Inhib	Inhib
Indole-3-glycolic	0.4	0.2	2
Indole-3-glyoxylic	? 0.1	ca 0	? 0.3
Indole-3-isobutyric	0.6 to 5.0 *	1.7	33 to 130 *
5-hydroxy-IAA	0.12	ca 0	4
7-hydroxy-IAA	0.06	ca 0	1.6
Indole-3-lactic	0.16	0.4	1.8
Indole-3-methylene malonic	0	0	0.3
Indole-4-acetic	1.0	1 to 6 *	2
7-Aza-IAA	50	6 to 100 *	ca 100
Indene-3-propionic	...	ca 0	3.5

5-Methyl and 6-methoxyindole; N-acetylindoxyl; oxindole-3-acetonitrile and -3-pyruvic oxime; oxindolene-3-acetonitrile and -3-pyruvic acid; 3-dehydrodioxindole; 3-methyldioxindole; imidazoleacetic acid, imidazoleacetonitrile and imidazoleacrylic acid were all inactive.

* Depending on which part of the curve is used for comparison.

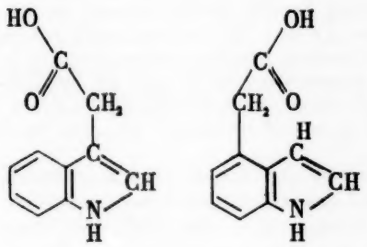
general "anti-auxin." Auxin antagonists should be defined by the specific bioassay in which they function.

It is satisfactory to observe that a number of the rules relating structure to activity, formulated originally from the aryl and aryloxy acids, hold good for indole derivatives also. Most striking is the effect of a hydroxyl group in lowering the activity. Whether in the side-chain, as in IGlycA and ILacA, or in the ring, as in 5-HO-IAA and 7-HO-IAA, the hydroxyl reduces the auxin activity for straight growth by at least 100 times. The activity for pea stem curvature is reduced 25 to 50 times. The similar example of mandelic, atrolactic and tropic acids has previously been discussed (20). One is tempted to deduce, modifying an earlier proposal of Veldstra, that the structure, and especially the ring, must not be made hydrophilic at any point other than the carboxyl, but can only be substituted by such lipophilic groups as Cl and CH_3. The inactivity of the oxindoles and the dicarboxylic acids would support this view, but, like so many other attractive proposals, it disagrees with an important part of the facts, namely the high activity of the phenoxy acids. For instance, 2,4-dichloro*phenoxy*acetic acid is about 50 times as active on pea curvature as 2,4-dichloro*phenyl*acetic acid. The phenolic oxygen, although it is in ether linkage, must certainly exert some degree of hydrophilic action. Thus the strong effect of a hydroxyl group, clear-cut as it is, remains unexplained.

Another characteristic cause of lowered activity, namely the introduction of a 2nd carboxyl, is exemplified by indolemethylenemalonic acid, compound no. 11 in this series. A third point, namely the inactivity of 5-membered ring compounds, is extended to three more instances by the imidazole derivatives here tested. Except for their ring structure these compounds would have been predicted to have good activity. Their complete inactivity makes a sharp contrast with the activity of the 6-membered pyridoxyacetic acids recently reported by Veldstra (30). Fourthly, the interchangeability of carbon and nitrogen in the ring, exemplified previously by the high activity of indene-3-acetic acid, receives additional strong support from the equally high activity of 7-Aza-IAA.

One of the early theories, especially emphasized by Wain, was that the side-chain must contain at least one hydrogen atom. The remarkably high activity of indoleisobutyric acid in pea curvature, and its real auxin activity on Avena, clearly disprove this view.

Finally, the very low activity of indole-4-acetic acid (I-4-AA), bears unfavorably on two current theories. In the first place, the position *ortho* to the side-chain is just as free in I-4-AA as in I-3-AA, so that the theory of Muir and Hansch (14) that activity depends on a free *ortho* position is satisfied in a qualitative way; however, quantitatively it offers no basis for a difference in activity of 100 times between the two compounds. In the second place, both I-4-AA and I-3-AA equally fulfill the proposed requirement of Jönsson (9) that the side-chain should be able to fold in such a way as to bring the carboxyl near the center of the compound ring system and in the same plane:



Hence this theory also provides no basis for a difference between the activities of the two acids of 100 times.

The only fair conclusion is that all the rules so far proposed are too simple, and none of them takes sufficient account of the varied features of an organic molecule. It is evident that there are rules, and strict ones, but their formulation is elusive. The interaction between the auxin molecule and its substrate evidently does not occur simply "at two points," or "in a plane," etc., but involves delicate gradations of electron density and spatial configuration, for the adequate description of which our present theories are too crude.

Summary

The activities of 20 indole derivatives and four related compounds have been determined in three bioassays; straight growth of Avena coleoptile sections, straight growth of Pisum stem sections and differential growth (curvature) of slit Pisum stem sections.

Indole itself promotes growth significantly but no evidence could be found for its conversion to IAA in vivo. Skatole inhibits growth in all three systems, and in pea stems the inhibition is clearly not competitive with IAA.

The four hydroxy derivatives, indolelactic, indoleglyoxylic and 5- and 7-hydroxy-IAA all have very low activity, which corresponds well with the low activity of hydroxy-aromatic acids. Six derivatives of oxindole and three of imidazole are inactive, the latter confirming the rule that compounds with 5-membered rings are inactive. Indolemethylenemalonic acid is virtually inactive, confirming the rule that dicarboxylic acids are generally inactive. Indenepropionic acid is much less active than indeneacetic acid.

Indole-3-isobutyric acid has low but real auxin activity on straight growth, and has activity close to that of IAA on curvature; it is therefore a true "shoot auxin."

Indole-4-acetic acid has only 1 to 2 % of the ac-

tivity of I-3-AA, which accords poorly with two theories of the structural requirements for auxin activity.

7-Aza-indole-3-acetic acid has high activity, approaching that of IAA, in all three systems.

It is concluded: 1) that auxin antagonists should be defined by the specific bioassay in which they function, 2) that the empirical rules relating structure of aromatic compounds to activity also hold good in the indole series, but 3) that the requirements for activity, though evidently stringent, are not well described by any of the recently proposed theories.

Excellent and faithful technical assistance was rendered successively by Mrs. Irma Slankis, Mrs. Berit Ørning, Mrs. Lisa Wilkinson, Mrs. Eileen Bladon, and Miss Irmgard Winkler. Thanks are due to Mr. W. R. Porter for carrying out the chromatographic analysis of indoleacetic acid. I wish also to thank Dr. Bruce B. Stowe for valuable discussions, for supplying three of the tested substances and for a critical reading of the manuscript.

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DEPTH CONTROLLED DEUTERON IRRADIATION OF LACTUCA SATIVA SEEDS. I. EFFECTS ON GERMINATION AND GROWTH[1]

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Although plant response to photon and particle irradiation is well studied, we are unaware of experiments involving an accurate control of the penetration depth of charged particles in multicellular organisms. Lateral restriction of such particles has been accomplished by micro-beam techniques (15). Longitudinal restriction can be accomplished only by fixing the kinetic energy of the particles before they reach the tissue to be irradiated. Preiss (11) has used the latter technique in obtaining the spatial distribution of intracellular yeast invertase. The charged particles were electrons with kinetic energies such that their trajectories ended well within the dimensions of the individual yeast cells.

The experiments described in the present paper are concerned with the germination and subsequent root growth effects produced by deuterons of various penetrating abilities in lettuce seed tissue. Analogous work by Busse and Daniels (2) involved placing corn seeds in a cathode ray beam with the embryos or endosperms facing the source.

The physiology of lettuce seed germination has been described in detail (for reviews see 4, 8, 14). Under the experimental conditions, the germination percentage of seeds kept in complete darkness after imbibition is usually less than 30. However, complete germination can be achieved when a short illumination is given briefly after imbibition. This photoreaction has a peak response at about 6700 Å, and can be reversed by less energetic photons (7000 to 7500 Å).

The embryo of the seed is surrounded by a number of layers. As described by Borthwick and Robbins (1) and by Evenari and Neuman (6) these include a pigmented fruit coat which contains large air spaces and is traversed by ridges of parenchymatous cells, a degenerated seed coat, and a dense endosperm two to three cell layers thick. The induction of germination is thought to be associated with layers surrounding the embryo (8), while subsequent growth is governed by factors in the embryo itself. Therefore, one would expect radiation induced changes to depend on the penetration depth of ionizing particles. Deuterons with energies up to about 4 Mev are excellent projectiles for such studies because their range in protein or cellulose is of the order of 100 μ or less (9). Alpha particles with about twice this energy meet these requirements; however the cyclotron beam currents are much smaller than those obtained by accelerating deuterons.

The aim of this work is twofold: to establish the usefulness of depth controlled irradiation as a tool in radiation biology and physiology on the macro scale, and, in the special case of the lettuce seed, to contribute to the understanding of germination. This report will deal mainly with the biological aspects; dosimetry, range measurements and calculations related to physical problems are discussed elsewhere (12).

MATERIALS AND METHODS

Lettuce seeds, var. Grand Rapids, obtained from Pieters-Wheeler were used exclusively in these experiments. The seeds were stored, prior to use, in a desiccator at 3° C. All particle bombardments were carried out on dry seeds in vacuo at room temperature. Immediately after irradiation batches of 50 to 100 seeds were deposited on wet filter paper in Petri dishes and kept in complete darkness at 25° C. The filter paper was thoroughly soaked with 2.5 cc of water. Light treatment, whenever desired, was given two hours after imbibition. Red light was obtained by the use of cellophane-wrapped fluorescent tubes giving an intensity maximum near 6500 Å. With a light intensity in the illumination plane of about 19 kiloerg/cm^2 × min, one minute of illumination was sufficient to promote complete germination of unirradiated controls. Germination percentage was noted, and root length was measured 48 hours after imbi-

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[2] Present address: Hebrew University, Jerusalem, Israel. Participation of S. Klein in this joint interdepartmental work was supported by grants from the Anna Fuller Fund and from the Yale University Atypical Growth Committee (American Cancer Society) to A. W. Galston.
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bition. In a few cases seedlings were planted and allowed to develop for 14 days.

In a few experiments low energy electrons were employed. These were accelerated in a multiple-gun rotating device designed specifically for the irradiation of large surface areas (11). Electrons from these guns could be given energies up to 5000 electron volts (e.v.), and their ranges in dry protein vary from about .01 μ (500 e.v.) to .57 μ (5000 e.v.) (3, 9, 11).

For deuteron treatment the analyzed beam of the 28-inch cyclotron at Yale University was used. In order to improve dose homogeneity in area the beam is spread by placing a 1-cm air equivalent of aluminum foil just beyond the beam analyzer. The beam is then circularly collimated to 2 cm^2 and enters the bombardment chamber which contains a cam-driven disc with counterbored depressions 1.27 cm in diameter. These depressions can be indexed into the beam path. The seeds were placed in these depressions and held in place by thin aluminum foils of known thickness, care having been taken that the seeds would not overlap. The beam must pass through this foil before hitting the seeds. Thus, it is foil thickness which determines the final energy of the deuterons and therefore their penetration depth in the lettuce seeds. It must be pointed out that in these experiments the number of particles passing through a foil is independent of foil thickness because none of the foils employed were thick enough to reduce the kinetic energies of the particles to zero. The number of deuterons per cm^2 applied to the seeds was found by measuring the charge picked up by the above mentioned disc either with a calibrated galvanometer or a beam current integrater.

The thickness of the aluminum foils was determined by weighing known areas on an accurate beam balance, and is given in mgm/cm^2 with a maximum error of 0.5 %. A 1.53 mgm/cm^2 aluminum foil is equivalent in stopping power to 1 cm of air at normal temperature and pressure (10).

Results

Effects of Radiation Penetration on Germination and Growth: For orientation the reader's attention is directed to figure 1 which shows the major effects of varying radiation dose and penetration depth. All the physical data concerning the treatments and the resulting germination and root lengths are given in table I. Mean deuteron ranges were calculated for proteinaceous material from the Bethe energy loss formula (7). For each aluminum foil

Table I

Energy and Range Data for the Treatments Given and Their Effects on Germination and Root Length

Aluminum foil thickness	Dose	Total radiation energy	Mean range in protein	Energy loss *		Germination after 48 hrs		Mean root length	
				Outside embryo	In embryo	Dark	Light	Dark	Light
mgm/cm²	*deuterons/cm²*	*erg/cm²*	*microns*	*erg/cm²*	*erg/cm²*	*%*	*%*	*mm*	*mm*
2.5	900×10^{11}	5.5×10^{9}	102	5.0×10^{7}	5.0×10^{8}	12	16	2	2
2.5	90 "	5.5×10^{7}	102	5.0×10^{6}	5.0×10^{7}	92	99	2	2
2.5	36 "	2.2×10^{7}	102	2.0×10^{6}	2.0×10^{7}	98	98	2	2
5.5	1×10^{11}	5.6×10^{5}	88	5.5×10^{4}	5.0×10^{5}	18	79	4.5	4.5
5.5	1×10^{10}	5.6×10^{4}	88	5.5×10^{3}	5.0×10^{4}	37	99	8.8	8.8
15.8	720×10^{11}	2.3×10^{9}	36	8.0×10^{7}	1.5×10^{9}	100	98	4.8	4.7
15.8	72 "	2.3×10^{7}	36	8.0×10^{6}	1.5×10^{7}	100	100	9.0	9.0
15.8	18 "	5.7×10^{6}	36	2.1×10^{6}	3.6×10^{6}	97	97	10	10
15.8	1 "	3.2×10^{5}	36	1.2×10^{5}	2.0×10^{5}	32	98	N	N
18.9	720×10^{11}	1.6×10^{8}	20	12.5×10^{7}	3.5×10^{7}	98	100	6.5	6.9
18.9	72 "	1.6×10^{7}	20	12.5×10^{6}	3.5×10^{6}	97	100	10	10
18.9	18 "	3.9×10^{6}	20	3.1×10^{6}	8.0×10^{5}	97	99	N	N
18.9	6 "	1.3×10^{6}	20	1.0×10^{6}	2.6×10^{5}	69	100	N	N
18.9	1 "	2.2×10^{5}	20	1.7×10^{5}	4.5×10^{4}	31	100	N	N
20.0	720×10^{11}	1.3×10^{8}	15	12.6×10^{7}	4.0×10^{6} **	98	95	7.8	8.1
20.0	72 "	1.3×10^{7}	15	12.6×10^{6}	4.0×10^{5} **	100	100	N	N
20.0	18 "	3.2×10^{6}	15	3.2×10^{6}	8.0×10^{4} **	100	98	N	N
21.5	720×10^{11}	7.2×10^{7}	7.5	7.2×10^{7}	0	99	100	N	N
21.5	72 "	7.2×10^{6}	7.5	7.2×10^{6}	0	86	99	N	N
21.5	18 "	1.8×10^{6}	7.5	1.8×10^{6}	0	53	98	N	N
21.5	1 "	1.0×10^{5}	7.5	1.0×10^{5}	0	33	98	N	N

* These figures are based on the measured protein equivalent for the fruit coat (5 μ) and for the seed coat + endosperm (11 μ) (12).

The numbers given are mean values for 2 to 4 Petri dishes, each containing 50 to 80 seeds.

N = normal root length (11 to 14 mm).

** Based on straggling calculation (Preiss and Klein (12)).

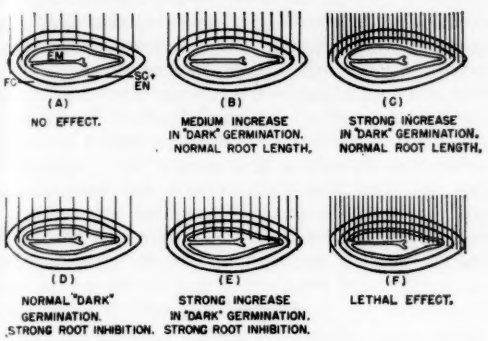

FIG. 1. The effects of deuteron irradiation on lettuce seed germination and subsequent root growth when applied to different depth levels: A to C—Deuterons penetrating to endosperm only. D to E—Deuterons penetrating deep into embryo.

A and D—Weak dose; B and E—medium dose; C and F—strong dose.

FC—Fruit coat; SC + EN—seed coat and endosperm; EM—embryo. The figures are not drawn to scale.

several doses were used, and the total incident energy in ergs/cm^2 is given in each case. This was obtained by multiplying the number of deuterons/cm^2 by the average energy of a deuteron after it passed through a particular foil. For each set of cyclotron runs seeds were placed in the cyclotron vacuum for the duration of a bombardment, but secured out of the deuteron beam. Others were kept at atmospheric pressure. Both of these control lots possessed the same properties, namely 98 to 100 % germination after red light irradiation, and 22 to 40 % germination when no red light was given. This latter "dark germination" rose to 60 % on two occasions, but did not alter the nature of the results obtained.

The data show that the light requirement for germination could always be overcome by some dose of deuterons, and furthermore that this dose had to be highest when the deuteron retarding foil was thickest (see the 21.5 mg/cm^2 data). However, in all cases more than 10^{11} deuterons/cm^2 are required to promote this reaction.

When the largest dose (9×10^{13} deuterons/cm^2) was administered through the thinnest foil (2.5 mgm/cm^2), allowing the deepest penetration, light as well as dark germination was greatly reduced. Only 5 % of these seeds which did not germinate during the first 48 hours after imbibition recovered after an additional cold treatment, while dark controls responded completely. No irradiations with lower doses applied through thicker foils were lethal, as is shown by the fact that the germination response to red light was the same as that of the controls.

If the reader compares the root length, dose, total energy, and deuteron range columns of table I, he will see that the important growth retarding factor is deuteron penetration depth in the seed. For example, 720 times as many deuterons must be applied through a 15.8 mg/cm^2 foil as through a 5.5 mg/cm^2 foil to produce the same reduction in root growth. The mean ranges are 36 and 88 μ respectively.

N in the table indicates normal root growth, which was about 11 mm for the dark and 14 mm for the light controls. This difference is not due to a different growth rate, but to a somewhat earlier germination of the light treated seeds (8).

It should be emphasized that germination and root growth effects can be separated by using the right combinations of foils and doses. Bombardment through heavier foils with doses between 10^{12} to 10^{13} deuterons/cm^2 will change dark germination percentage without effect on root length. However, a deeper penetration with smaller doses (10^{11} deuterons/cm^2) will result in much shorter roots but the dark germination of the seeds will remain as low as that of the controls.

Treatment with 4500 electron volts did not change either root length or germination percentage. Since electrons with the energy chosen would penetrate into protein up to 0.5 μ, it can be assumed that they were stopped in the outermost cell layer of the fruit coat. If these seeds were given a high electron dose while on a bronze plate, a sharp image was formed, indicating that a major portion of the incident electrons was not deflected.

MORPHOLOGICAL EFFECTS OF THE IRRADIATION ON THE SEEDS: The various deuteron treatments caused different degrees of visible damage. With a 2.5 mg/cm^2 foil and doses from 3.6×10^{12} to 9×10^{13} deuterons/cm^2 the cotyledon which intercepted the incoming beam was damaged and blackened to a depth of 100 to 120 μ. Hand-cut sections of the damaged cotyledons revealed a well defined and wavy damage border, the border more or less following the contours of the seed coat. With heavier foils (15.8, 18.9, 20 mg/cm^2) the damaged layer is not continuous, but reddish brown stripes traverse the cotyledons. With increasing foil thickness the stripes get thinner and shallower, and with a 20 mg/cm^2 foil, damage is restricted to a depth of 20 to 25 μ. It has not been established if this discolored area indicates the actual penetration depth of the deuterons. It is possible that only part of the cell has to be hit to cause damage to the whole cell; an increase of a few μ in penetration would, therefore, push the damage-border 10 to 20 μ deeper into the seed; also it may well be that the damage done to one cell spreads to the neighboring ones. Furthermore, the degree of visible damage is dependent on dose. A dose of 10^{11} deuterons/cm^2 does not cause any visible discoloration in spite of its pronounced effect on root growth when applied through thin foils.

Another phenomenon observed after deuteron treatment was the occurrence of bright red spots on the filter paper surrounding the germinating seeds. Pigment formation was strongest with treatments which allowed moderate root growth. When root growth was much depressed or normal only a very

faint coloration occurred. The irradiation of isolated fruit coats and embryos revealed that this pigment results from embryo damage, and did not originate in the fruit coat. The same color appears faintly when rootlets of untreated seedlings were slightly injured and left to germinate for one or more days. To our knowledge this particular irradiation effect has never been reported.

The pigment is insoluble or only slightly soluble in many polar and nonpolar solvents such as water, alcohol, normal and tertiary butyl alcohol, carbon tetrachloride and chloroform, but is soluble in N, N-dimethylformamide and dimethylsulfoxide. The spots are stationary when run in an electrophoresis apparatus in 0.5 M phosphate buffer at pH 5. The visible spectrum of the pigment on the paper shows a peak between 4950 and 5300 Å. The data indicate that this pigment might be identical with the one found by Swanson et al in *Helianthus tuberosus* tuber discs and other Compositae after treatment with 2,4-dichlorophenoxyacetic acid (13).

Very frequently the embryo pierced the seed coats at the upper end while the root tip remained embedded, and where embryo growth had occurred the seed coats adhered to the base of the root (2). This effect was absent when the combination 5.5 mgm/cm^2 foil and 10^{11} deuterons/cm^2 was used, as well as in all experiments employing 21.5 mgm/cm^2 foils.

So far only one experiment, in which 1.8×10^{12} deuterons/cm^2 were applied through a 20 mgm/cm^2 foil, was carried out to test a possible far-red reversibility of the deuteron induced germination. No such reversibility could be found when the deuteron-exposed seeds were exposed to 10 minutes of far-red light.

The deuteron treatment does not seem to prevent the development of non-irradiated tissues. Table II shows the mean length of main roots, hypocotyls and the oldest leaf of 9 plants after a growth period of 12 days. The developmental stage of these plants was about the same, with the 1st three leaves emerged. Seeds which were deeply penetrated by the deuterons gave rise to plants which had one normal cotyledon; the other was either dead or very small and heavily damaged. After the more shallow treatments the affected cotyledon was somewhat smaller than the unaffected one, curled, and still green. The data indicate a larger effect of penetration depth of dose: root lengths of plants from seeds treated with 3.6×10^{12} deuterons/cm^2 applied through a 5.5 mgm/cm^2 foil is less than after a treatment with 7×10^{13} deuterons/cm^2 through a 20 mgm/cm^2 foil. Secondary root formation from the hypocotyl was observed only after treatments using 2.5 mgm/cm^2 foils. In this case portions of the main root did not recover at all and a secondary root from the hypocotyl replaced it.

TABLE II

LENGTH AND FRESH WEIGHT OF 14-DAY-OLD LETTUCE PLANTS FROM DEUTERON BOMBARDED SEEDS *

FOIL THICKNESS	DOSE	LENGTH		FRESH WEIGHT OF WHOLE PLANT
		ROOT	LARGEST LEAF	
mg/cm²	*deuterons/cm²*	*mm*	*mm*	*mg*
0	0	93	42	164
0	0	89	39	140
2.5	3.6×10^{12}	53	14	39
20.0	7.2×10^{13}	62	25	73
20.0	7.2×10^{12}	93	46	175

* Mean values of 9 plants per treatment.

DISCUSSION

The data, interpreted on the basis of mean deuteron ranges in protein, show that radiation applied at different depths in the seed elicits different germination and growth responses. However, penetration ranges in protein cannot by themselves be used for interpretation on a morphological basis because, as described earlier, the layers surrounding the embryo are structurally inhomogeneous, and contain relatively large air spaces. Therefore, the energy loss of deuterons passing through these layers had to be determined experimentally. Our measurements (12) showed that the fruit coat was equivalent in stopping power to 5 ± 1 μ of protein, while the combined seed coat and endosperm was found to be equivalent to 11.5 ± 1 μ of protein.

Taking these data into account the energy loss within and without the embryo of the seed could be calculated. These values have been listed in table I.

GERMINATION: Deuterons applied through a 21.5 mgm/cm^2 foil are stopped in the endosperm, since the mean range is only 7.5 μ in proteinaceous material. The absence of cotyledon scarring with the highest dose applied is additional evidence that the embryo itself is not hit. However, dark germination is always raised, often to 100 %, from which one can conclude that only the layers surrounding the embryo have to be affected to overcome dark dormancy. Results obtained by other workers (5, 6, 14) are in accord with this interpretation. To increase dark germination with this treatment, a minimum energy of about 2×10^6 erg/cm^2 is required. If, however, irradiations were made through thinner foils, in which case the deuterons penetrated into the embryo, less energy (10^6 erg/cm^2) was required in the outer seed layers to give equivalent germination percentages. The reason may be that in the first case deuteron energy is not lost throughout the entire endosperm thickness, as in the latter case. However, the possibility that embryo irradiation may moderate this energy requirement cannot be excluded without a more sophisticated argument (12).

GROWTH: The data in table I show that root growth inhibition occurred only if deuterons reached the embryo. Obviously the penetration depth of the radiation into the embryo is of primary importance. This is immediately apparent when the 5.5 mg/cm^2

foil data are compared with those taken with thicker foils, for which the root lengths are about the same.

For doses greater than or equal to 3.6×10^{12} deuterons/cm^2, penetrating deeply into the embryo, root length after two days was very small, and many of the roots were dead 14 days later. The scarring produced by higher doses of less penetrating deuterons showed that most damage was done to the cotyledons, whereas with deeper penetration, both cotyledon and radicle were damaged. Although our data will not permit the distinction between effect of cotyledon and radicle damage on later root growth, it would appear that the radicle damage is the more important function. This is because the part of the endosperm which surrounds the radicle is three cell layers thick, and may act as a shield to all but the most energetic (deeply penetrating) deuterons.

It is noteworthy that a 10^{11} deuteron/cm^2 dose applied through a thin foil will diminish root length without changing the germination properties of the seed. This suggests that with radiation of this type, a kind of micro-surgery can be carried out inside the organism without changing the gross physiological behavior of the outer layers.

THE PHOTO-RECEPTOR: The fact that deuteron bombardment can overcome dark dormancy does not allow one to draw conclusions concerning the red light mediated photo response. However, no deuteron treatment resulting in less than 100 % dark germination impaired this response. This means that if the primary red light receptor is within the range of these deuterons, it must be a relatively small target in order for its inactivation probability to be unmeasurable, even with such large doses as were employed in our work. Such doses would readily inactivate protein molecules and large polypeptides such as gramicidin.

SUMMARY

The effect of depth penetration of various doses of deuterons on lettuce seed germination and subsequent growth was investigated. Differences between irradiation of the coats and the endosperm, and of the embryo itself were found.

1. Irradiation of the coats and the endosperm alone increases the germination of the seeds in the dark. Deeper irradiation into the embryo moderates the dose requirements for germination.

2. Root length inhibition occurred only when the embryo itself was irradiated. The energy requirement for root inhibition was found to be inversely related to depth of penetration in the embryo. With small but deeply penetrating doses root length was inhibited without changing the germination pattern.

3. The effect of red light on germination could not be influenced by irradiation. From this it was concluded that the primary photo receptor was a relatively small molecule.

4. The deuteron induced dark-germination is not reversible by far-red light.

5. A red pigment diffusing from the seedling after irradiation is described, as well as other radiation-responses.

The authors would like to express their appreciation to Dr. A. W. Galston and Dr. E. C. Pollard for criticism and interest in this work, and to Dr. L. V. Barton from the Boyce Thompson Institute for the spectral analysis of the pigment spots.

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DEPTH CONTROLLED DEUTERON IRRADIATION OF LACTUCA SATIVA SEEDS. II. ENERGY LOSS IN THE OUTER SEED LAYERS[1]

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In a previous paper (2) the germination and subsequent growth of deuteron-irradiated lettuce seeds was described, with special emphasis on the influence of penetration depth. It was shown that depth of penetration is the predominant factor in eliciting the effects described, i.e., germination percentage and root length are affected at different depth levels. The general phenomena observed in our experiments do not differ essentially from effects of radiation described by other workers. It was possible, however, to pin down the radiation effects locally, and to make a more detailed analysis of the radiation data by following the energy loss through the various layers. The results obtained suggest that this application of techniques of deuteron bombardment to a biological problem is of value in the study of multicellular organisms where a localization of certain physiological activities is required.

Since range accuracy and dosimetry are of utmost importance in work of this kind these aspects will be discussed in this article. A method by which the energy lost in the outer seed layers was determined with the deuterons themselves will be described. It will be shown that germination induced by ionizing radiation can be ascribed solely to effects produced on the endosperm.

DETERMINATION OF RADIATION ENERGY LOSS IN THE OUTER SEED LAYERS: *Determination of the relative stopping power of the outer seed layers with respect to air (N.T.P.)*: In order to obtain the mean ranges of deuterons in an average protein of density 1.3, the rate of energy loss per unit distance was calculated with the Bethe-Bloch formula, and a numerical integration of the reciprocal of this rate was performed. The average excitation potentials used for carbon, hydrogen, nitrogen, and oxygen were those listed by Pollard (3). It was found that the mean range of deuterons in proteinaceous material, measured in microns, is five times their mean range in aluminum, expressed as mgm/cm^2. In stopping power, 1.53 mgm/cm^2 of aluminum is equivalent to 1 cm of air at N.T.P. (4).

Values for protein were used when calculating depth of penetration into the embryo. They could not, however, be applied directly to the layers surrounding the embryo. The problem arose not so much from the different chemical constitution of these layers but from their inhomogeneous structure. The fruit coat, the external part of the seed, has a very uneven surface and contains large air spaces. The endosperm, the layer closest to the embryo, is much thinner but more dense. Therefore, direct measurement of the thickness of these structures would not give an indication of their ability to retard deuterons. A method had to be found whereby their stopping powers relative to air or aluminum could be measured. With this information their thicknesses could be expressed in equivalents on a calculated average protein scale.

A range cell containing the usual variable air pressure chamber and continuously evacuated beam pick-up chamber was constructed. The variable pressure chamber, which is 6.9 cm long, was isolated from the cyclotron vacuum and the beam pick-up vacuum by 5.5 mgm/cm^2 and 11.0 mgm/cm^2 aluminum foils respectively (equivalent in stopping power to 3.6 cm and 7.2 cm of air). Since a 4-Mev deuteron is stopped by about 15 cm of air at N.T.P., these foils, together with the air column in the range chamber at somewhat less than atmospheric pressure, stopped the beam. A fixture containing five no. 68 drill holes was placed in the chamber between the foils, either with the holes open, or with fruit coats or combined seed coats and endosperms clamped in place so as to

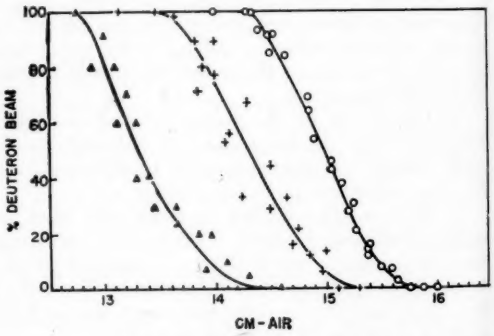

FIG. 1. "Number-distance" curves used to measure the stopping power of the outer seed layers relative to that of air. ○—Air, corrected to N.T.P. +—Air plus the fruit coat. △—Air plus the endosperm and seed coat. The mean range of 15 cm (air curve) corresponds to a kinetic energy of 4.1 Mev for the bombarding deuterons.

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block them. Although the amount of beam current coming through the five holes was small it could be read on a sensitive galvanometer which was not quite critically damped. The three range curves in figure 1 were obtained by measurement of the change in detected current as the air pressure was varied. The horizontal separations were measured at several ordinate values. The average thickness of the seed coat–endosperm was found to be equivalent to 11.5 ± 1 μ of 1.3 density protein; that of the seed coat alone was found to be 5 ± 1 μ of protein. This latter is a mean value, for the range curve of the fruit coat tends to approach the range curve of air near the tail. This is an indication that a small number of deuterons traverse sections equivalent to less than 5 μ of protein. Furthermore, when cotyledon damage was observed, the damaged area was (for the least energetic deuterons which reached this region) traversed by narrow strips of undamaged tissue. Therefore, since these followed fruit coat ridges, it must be concluded that the ridges represent much more than 5 μ of protein. However, the only effect of taking the average value for Bragg curve calculations is the prediction of too high an energy loss in the dense inner regions of the seed. Since our basic aim is to show that deuteron irradiation of the embryo is not required to overcome the dark dormancy of the lettuce seed, our argument will therefore be more conservative than necessary.

Construction of Bragg curves for a protein absorber: If a monoenergetic beam of ionizing particles passes through a layer of matter, the particles emerge with a certain distribution of energies about some mean value, since all of them do not produce exactly the same number of ionizations and excitations. Therefore, the distances they travel before being stopped in an absorber will likewise be distributed about a mean value. The width of this distribution is a measure of the "straggling" of these particles.

If the aim of a radiation experiment is, as in this study, to find out if or to what extent a physiological function is located in a certain layer of an organism, the straggling phenomenon may place a serious limitation on the depth resolution. This is especially true when the thickness of the layer to be investigated is comparable to the straggling distance. Since the protein-equivalent thickness of the endosperm is only about 10 μ, special consideration had to be given to this problem.

In figure 1, the distance along the range axis, from the point where the detected current just begins to fall to the point where it is just detectable was measured. This distance represents about 1.3 cm of air at N.T.P. or about 10 μ of average protein (density 1.3). A deuteron which can traverse 1.3 cm of air has a kinetic energy equal to about 0.8 Mev. Since the sloping portion of a "number-distance" curve can be approximated by a normal distribution function about the mean range, R microns of protein (1), it follows that a plane, normal to the deuteron beam at (R – 5) microns, can be considered as a source of deuterons with energies varying from 0.0 to 0.8 Mev. From this it follows that a normal density function, the mean of which is at R microns, can be used as an energy weighting function. This procedure was carried out in constructing Bragg curves for a protein absorber, some of which are shown in figure 2. The wide energy distribution undoubtedly allows for possible cyclotron beam energy inhomogeneity. The assumption was made that straggling was produced to the same degree among the original 4.1-Mev deuterons traversing a thin aluminum foil and penetrating deep into the lettuce seed, as among those traversing a thick aluminum foil and penetrating a shorter distance into the seed. This permits a single straggling correction for all mean ranges, since only atoms of low atomic number are involved, both in the foil and in the organism.

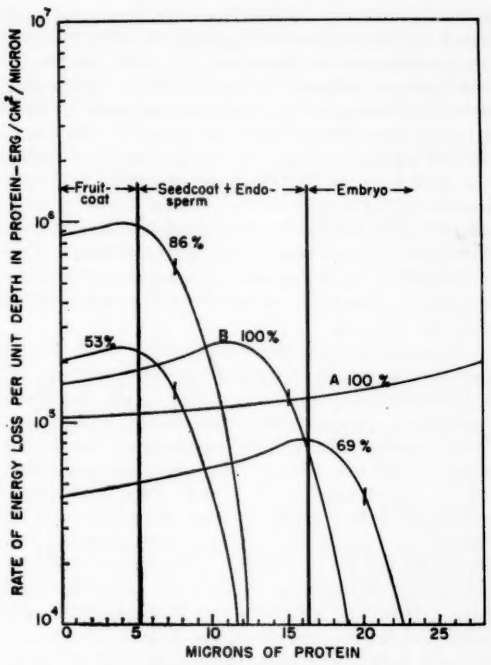

FIG. 2. Bragg curves for a protein absorber, obtained for the five deuteron treatments listed in table I. The boundaries of the outer seed layers have been included. Germination percentages have been indicated on each curve. Mean range of the deuterons are indicated by vertical dashes. The mean range for curve A (100 %) is at 36 μ.

The Bragg curves show that the energy loss rate varies from about 7×10^{-9} n ergs/cm²/micron in the 5th micron beyond the mean range to 1.25×10^{-7} n ergs/cm²/micron in the 5th micron before this point. (n = the applied dose in deuterons/cm².) At the mean range, it is about 8×10^{-8} n ergs/cm²/micron of protein. The total integrated energy loss beyond the calculated mean range is about 10^{-7} n ergs/cm².

As an illustration of the deuterons with a mean penetration of 7.5 μ in protein material, 2×10^{12} deuterons lose 2×10^{6} ergs/cm^2 in the seed, 2×10^{2} ergs/cm^2 of which appear beyond the mean range. These data are taken from the 53 % germination curve of figure 2. Only 1.4×10^{4} ergs/cm^2 are lost in the 5th micron beyond the mean range. No visible embryo damage was seen with the highest doses applied. If 2×10^{12} deuterons/cm^2 penetrate to a mean depth of 15 μ, about 2×10^{5} ergs/cm^2 out of a total of 4×10^{6} ergs/cm^2 appear beyond this point. This treatment causes a very slight but visible embryo damage, which is due to a small amount of energy straggling past the 16.5 μ depth. (See fig 2.)

Localization of the Seed Region Involved in Deuteron-induced Germination: It was shown (2) that when deuterons were stopped in the endosperm a minimum dose of about 10^{13} deuterons/cm^2 completely overcame the light requirement for lettuce seed germination. When the deuterons penetrated part of the embryo itself, a dose about an order of magnitude lower produced the same effect. The former case establishes the fact that ionizations do not have to occur in the embryo to initiate germination, i.e., it is *sufficient* to irradiate only the endosperm. However, the latter case in which both endosperm and embryo are irradiated suggests that it may not be *necessary* to irradiate the endosperm. A more critical analysis will show that irradiation of the endosperm is both *necessary* and *sufficient* to obtain an increase in dark germination.

In figure 2 various Bragg curves have been plotted for a protein absorber. The boundaries between the lettuce seed layers, as previously established, have been included. The curves represent the energy loss rates for the five deuteron irradiations, each resulting in a different germination percentage, which are listed in table I.

For the mean penetration ranges here represented, germination can always be raised to 100 % by increasing the number of deuterons per square centimeter (dose). The 53 % and 86 % curves, for which the mean range is 7.5 μ, illustrate this. No counter effect, even with a dose as high as 10^{14} deuterons/cm^2, was ever observed. A low germination due to a lethal effect occurred when penetration depths were of the order of 100 μ.

Table I

Irradiations for Which the Bragg Curves in Figure 2 Were Constructed

Deuterons/cm^2	Mean penetration	Dark germination
1.8×10^{12}	7.5 microns	53 %
6.0×10^{11}	20.0 microns	69 %
7.2×10^{12}	7.5 microns	86 %
1.8×10^{12}	36.0 microns	100 % (A)
1.8×10^{12}	15.0 microns	100 % (B)
0	0	30 %

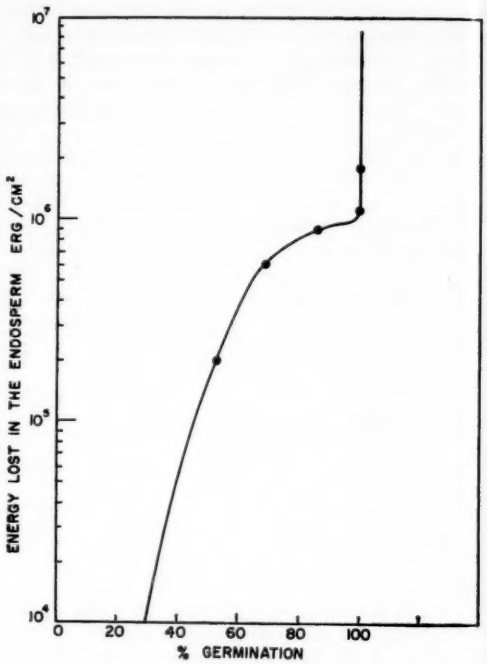

Fig. 3. Energy loss in the endosperm as a function of germination percentage. (Integrals of the five sample Bragg curves of figure 2 from 7 to 16.5 μ.) Such a single-valued function would not be obtained for integrals over the fruit coat or embryo regions.

That the endosperm is the seed region responsible for deuteron induced germination is deduced as follows. The sensitive seed region must lie partly or wholly within the regions bounded by the origin and the range axis intercepts of the Bragg curves (fig 2) since deuteron induced germination occurs in each case. Since germination is a function of dose, it follows that it must also be a function of the energy lost throughout this sensitive region. Furthermore, it is a non-decreasing function of the energy lost in the sensitive region, because no dose was ever high enough to decrease germination even when the mean range of the bombarding deuterons was 36 μ. Both dose (number of deuterons/cm^2) and deuteron energy determine the energy loss per μ in the sensitive layer and therefore the energy loss throughout that layer. Therefore, no Bragg curve associated with a certain germination percentage should ever lie above another with a higher percentage in the region where deuterons induce germination. In other words, whenever germination percentage cannot be represented by a non-decreasing function of the energy loss in a particular layer of the seed, this layer must be discounted as far as germination effects produced by ionizations are concerned.

In the region of the fruit coat total energy losses (integrals) for the 53 % and 86 % curves are higher

than those for 100 % curves. Since there exists no non-decreasing functional relationship between germination percentage and energy loss in this region, ionizations in the fruit coat cannot be responsible for the induced germination. Likewise, in the region of the embryo there is a 69 % germination curve which lies above a 100 % curve. (In both of these cases visible embryo damage could be seen.) Therefore, germination increase cannot be ascribed to ionization effects in the embryo. However, the required functional relationship between germination percentage and energy loss is satisfied in the endosperm region (fig 3). The points on this curve were obtained by integrating the five rate curves of figure 2 from 7 to 16.5 μ.

It can be concluded that a sub-surface shell-like region of the seed exists where ionizations induce germination. The measured values of the stopping powers of the outer seed layers relative to a standard protein absorber allow us to identify this region as the endosperm.

Summary

Low energy deuterons were used as probes to establish which morphological entities of the lettuce seed are responsible for ionization-induced germination. For this purpose, the deuteron retarding ability of the fruit coat and seed coat–endosperm was measured with a range cell. Bragg curves for irradiations at various deuteron energies were constructed. It was found that it is *necessary* and *sufficient* to irradiate the endosperm to overcome the dark dormancy of lettuce seeds.

The authors would like to express their appreciation to Professors E. C. Pollard and A. W. Galston and Drs. Harry Rappaport and H. J. Morowitz for their criticism and interest in this work.

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GIBBERELLIC ACID, PART VI. THE BIOLOGICAL ACTIVITY OF ALLOGIBBERIC ACID AND ITS IDENTITY WITH GIBBERELLIN B[1]

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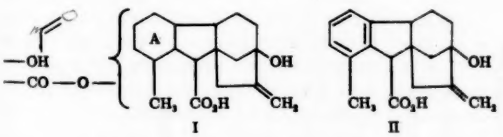
Mild acid hydrolysis of gibberellic acid (I) yields *allo*gibberic acid (II) (5, 6) with aromatization of ring A and evolution of carbon dioxide. Cross (5) has presented evidence that *allo*gibberic acid is identical with gibberellin B, a substance obtained from culture filtrates of *Gibberella fujikuroi* by Yabuta et al (10) along with gibberellin A, and also obtained (11) by treatment of gibberellin A with mineral acid at low temperature.



Gibberellin B was reported by Yabuta et al (10) to produce elongation of rice seedlings (i.e., the "bakanae" effect) in the same way as gibberellin A, but that it was markedly less active. Cross (5), on the basis of data on growth of dwarf peas supplied by one of us, reported that *allo*gibberic acid was biologically inactive. Stowe and Yamaki (8) have drawn attention to this apparent contradiction, and have suggested that the presumed identity of *allo*gibberic acid and gibberellin B should be reconsidered. As they rightly point out, it is of some importance to know whether or not ring A of gibberellic acid is necessary for biological activity.

In this paper we present in greater detail the evidence for the identity of *allo*gibberic acid and gibberellin B and report further evidence that *allo*gibberic acid is inactive in inducing shoot extension. In addition, since Stowe and Yamaki (8) have also stated that gibberellin B may stimulate root growth, whereas gibberellic acid and other gibberellins usually do not, we have examined the effects of *allo*gibberic acid on root growth.

Methods and Materials

Dwarf Pea Test: The materials to be examined were applied to 14-day-old dwarf pea seedlings, var. Meteor, in doses of 0.01 to 10.0 μg in 2 μl ethanol solution. The height of the plants was measured at the beginning of the experiment and 3, 7 and 10 days afterwards. Sixteen replicates of each treatment were used. The method has been described in greater

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detail previously. A dose of 0.01 μg gibberellic acid produces a detectable growth response (2).

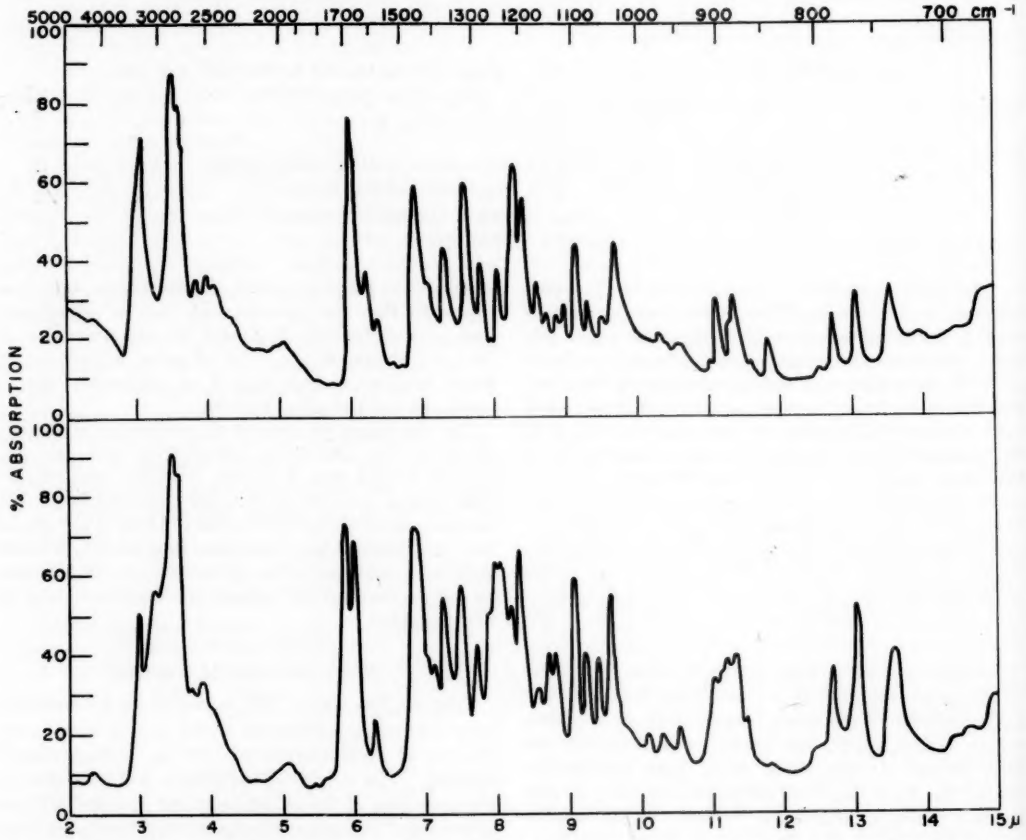
CRESS ROOT GROWTH TEST: The method used was that of Audus (1) modified as previously described (4). Six replicates of 20 seedlings were used for each treatment.

MATERIALS: An improved preparation of *allo*gibberic acid was used. Gibberellic acid (100 mg) was dissolved in water (67 ml) at 50° C, the solution was cooled to room temperature, treated with dilute hydrochloric acid (33 ml, 3 *N*) and allowed to stand at room temperature for 4 days. *allo*Gibberic acid hydrate separated in needles (58 mg, 66 %), melting point 125 to 130° C (loss of water) resetting ca. 140° C and remelting 195 to 197° C. Found: (dried at 20° C over phosphorus pentoxide) C, 71.4; H, 7.5. $C_{18}H_{20}O_3 \cdot H_2O$ requires C, 71.5; H, 7.3 %). On drying in vacuo at 100° C over phosphorus pentoxide the water was removed giving anhydrous *allo*gibberic acid melting point 195 to 197° C (Found: C, 75.8; H, 7.0. Calc. for $C_{18}H_{20}O_3$: C, 76.0; H, 7.1 %).

*allo*Gibberic acid hydrate crystallized from toluene (half of the solvent was distilled to ensure removal of water) in needles of the anhydrous acid, melting point 198° C (Found: C, 76.0; H, 7.1 %). Further crystallization of some specimens from benzene-methanol (plates) and toluene raised the melting point to 200 to 203° C $[\alpha]_D^{25} - 84° \pm 3$ (c, 0.94 in ethanol) (Found: C, 76.2; H, 7.1 %).

The infra-red spectra of *allo*gibberic acid and its monohydrate were identical (CO band, 1735 cm^{-1}, monomeric carboxyl) in solution in dioxan but the spectra of the crystalline compounds in Nujol mulls showed characteristic differences (figs 1 and 2) owing to different modes of hydrogen bonding in the solid state. Anhydrous *allo*gibberic acid showed a double peak at 1715 and 1679 cm^{-1} in the double bond stretching region and hydroxyl absorption at 3460 and 3160 cm^{-1}, whereas the hydrate showed a single carbonyl band (1700 cm^{-1}), a band at 1636 cm^{-1} (? water) and bands at 3350 and 3480 cm^{-1} (hydroxyl groups).

Three samples of *allo*gibberic acid were tested in the work reported in detail below: (a) Needles, melting point 198 to 200° C $[\alpha]_D^{17} - 73° \pm 3$, (c, 1.115 in ethanol) obtained from the standard preparative



FIGS. 1 and 2. Infra-red spectra in Nujol: 1 (*top*) *allo*gibberic acid hydrate and 2 (*bottom*) *allo*gibberic acid.

TABLE I

R_f VALUES OF SPOTS ON CHROMATOGRAMS OF SOLUTIONS OF GIBBERELLIC ACID AND *allo*GIBBERIC ACID PREPARATIONS

COMPOUND	WEIGHT /SPOT (μG)	TEMPERATURE OF DEVELOPMENT							
		18 TO 20° C				28 TO 30° C			
Gibberellic acid									
Pure	10	..	..	.40	..	.02	.34	.42	..
Aged aqueous solution	10	.02	.36	.43	..				
*allo*Gibberic acid									
(a)	200	..	.35	.41	.58	..	.32	.38	.56
(b)	600					..	..	..	.58
(b)	1200					..	.36	.40	.58
(c)	1200					..	..	..	.56

grade (above) of *allo*gibberic acid hydrate by crystallisation from toluene. (b). Plates, melting point 200 to 202° C $[\alpha]_D^{20} - 84° \pm 3$, (c, 1.10 in ethanol) obtained by recrystallization of (a) from benzene-methanol (Found: C, 76.3; H, 7.2). (c). Needles, melting point 198-201° C $[\alpha]_D^{21} - 84° \pm 3$, (c, 1.12 in ethanol) obtained as follows: (b) was heated for one hour under reflux with *N* sodium hydroxide solution and the product obtained by acidification and filtration crystallized from toluene. *allo*Gibberic acid is stable under these conditions of alkaline hydrolysis (5) which decompose gibberellic acid.

Specimens (a), (b) and (c) of *allo*gibberic acid, gibberellic acid, and an aqueous solution of gibberellic acid which had been kept at room temperature for 7 weeks were chromatographed on Whatman's no. 3 MM paper (18¼ × 22½ inches) using the solvent system *n*-butanol : ammonia (d 0.880) : water (4 : 5 : 1; descending technique). After equilibration of the paper for 1 to 16 hours in the presence of the lower phase (see below), development with the upper phase for 6 to 8 hours, and drying, spots were made to fluoresce in ultra-violet light by spraying with 5 % ethanolic sulphuric acid followed by heating for a few minutes at 80° C (7).

*allo*Gibberic acid gave very weakly fluorescent brownish spots, not visible unless comparatively large amounts of material were present. Gibberellic acid (violet spot) was detected at a minimum of 0.5 μg per spot. Results are summarized in table I.

When the temperature of development was 18 to 20° C, gibberellic acid gave a spot with R_f ca. 0.40, whereas the aged aqueous solution of gibberellic acid gave, in addition to the above spot, one with R_f 0.02, caused by a degradation product which will be described in a later paper, and an unidentified spot with R_f ca. 0.35.

When development was carried out at 28 to 30° C, gibberellic acid gave results similar to the aged aqueous solution of gibberellic acid; this effect was sometimes obtained with development at 20° C when the time of preliminary equilibration of the paper had been extended to 16 hours. Specimen (c) of *allo*gibberic acid gave a spot with R_f ca. 0.56, and no spot corresponding to gibberellic acid was detected. Specimen (a) gave spots with R_f values corresponding to *allo*gibberic acid, gibberellic acid and the unidentified material with R_f ca. 0.35. The content of gibberellic acid in specimen (a) was roughly estimated as 0.3 %. Specimen (b) of *allo*gibberic acid contained only traces of gibberellic acid, not always detectable even at 1200 μg of material per spot.

RESULTS

EFFECTS ON PEA SHOOT GROWTH: The result quoted by Cross (5) was obtained with Meteor peas grown in nutrient solutions, the *allo*gibberic acid being included in the nutrient solution. Two samples were used—anhydrous *allo*gibberic acid, at concentrations of 42.8 and 4.8 μg/ml and *allo*gibberic acid hydrate at 13.75 and 1.5 μg/ml. Neither induced increased growth; indeed the higher concentrations were toxic, though this was attributed to the use of alcohol (0.25 % v/v) in making the solutions.

In later tests, where *allo*gibberic acid was applied to the leaves, increases in growth were obtained with some samples. This only happened where large doses (10 to 100 μg/plant) were used and never suggested that *allo*gibberic acid was of an activity exceeding 0.1 % that of gibberellic acid. However, in view of the element of uncertainty, the three samples listed under Methods and Materials above were tested at the same time at four dosage levels. The results are presented in table II. Sample (a) of *allo*gibberic acid produced a significant growth increase only at the highest dose (10 μg/plant). The two samples which had been most rigorously purified, (b) and (c), produced no growth increases. It therefore seems most probable that the increase produced by sample (a) was due to the contamination with gibberellic acid demonstrated chromatographically (see Methods and Materials). Pure *allo*gibberic acid had less than 0.1 % the activity of gibberellic acid, i.e., to all intents and purposes is inactive. The apparent inhibition of growth caused by the 0.01 μg dose of sample (a) can probably be ignored as it could well occur by chance in an experiment of this size.

TABLE II

MEAN GROWTH INCREMENT (MM) OF METEOR PEAS 10 DAYS AFTER TREATMENT

TREATMENT	DOSE (μG)			
	0.01	0.1	1.0	10.0
*allo*Gibberic acid: (a)	67 *	76	81	109 **
" " (b)	73	77	88	96
" " (c)	82	77	90	86
Gibberellic acid	104 **	112 **	165 **	205 **
Untreated control		83		

* Significantly different from control at 5 % level.
** Significantly different from control at 1 % level.
Standard error = 5.1.

TABLE III

MEAN LENGTH (MM) OF ROOTS OF CRESS SEEDLINGS

TREATMENT	CONCENTRATION (μG/ML)		
	10	1	0.1
Gibberellic acid	29.6	30.2	30.0
*allo*Gibberic acid: (a)	16.6 *	29.1	31.0
" " (c)	19.8 *	33.2	33.6
Untreated control		32.1	

* Significantly different from control at 1 % level. Standard error = 1.65.

EFFECTS ON CRESS ROOT GROWTH: The results of a cress root growth test are given in table III. Data for all treatments except controls are means of 6 replicates of 20 seedlings; for the untreated controls 24 replicates of 20 seedlings were used. Gibberellic acid had no effect on root growth in this dosage range, as previously reported (4). At no dose did either sample of *allo*gibberic acid increase root growth; at the highest concentration (10 μg/ml) both samples significantly reduced root growth.

This result raised one further possibility which we have tested by experiment. The surprising lack of effect of gibberellic acid on growth of intact roots could be explained if some natural "gibberellin-like hormone" were always present in optimal quantities. The inhibitory effects of *allo*gibberic acid could then in turn be attributed to its structural similarity to the gibberellins, only ring A being different. If this were so the inhibition caused by *allo*gibberic acid should be reduced by gibberellic acid. The data presented in table IV show that this is not so; gibberellic acid is without effect on cress root growth alone or in the presence of *allo*gibberic acid.

TABLE IV

MEAN LENGTH (MM) OF ROOTS OF CRESS SEEDLINGS GROWN IN MIXTURES OF GIBBERELLIC ACID AND *allo*GIBBERIC ACID (SAMPLE c)

*allo*GIBBERIC ACID μG/ML	GIBBERELLIC ACID (μG/ML)			
	0.0	0.1	1.0	10.0
0.0	27.5	24.1	28.0	27.1
0.1	27.9	23.8	23.9	25.3
1.0	26.2	24.8	22.8	22.9
10.0	19.5 *	20.2 *	15.2 *	17.5 *

* Significantly different from untreated at 1 % level. Standard error = 2.00.

ANALYSIS OF VARIANCE

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Gibberellic acid	3	43,631	14,544
*allo*Gibberic acid	3	397,207	132,402 **
Interaction	9	72,863	8,096
Residual error	80	672,227	8,403
Total	95	1,185,928	.....

** Variance ratio significant at 0.1 % level; other variance ratios not significant.

IDENTITY OF ALLOGIBBERIC ACID AND GIBBERELLIN B: An authentic specimen of gibberellin B received from Professor Sumiki melted at 125° C, reset at 135 to 140° and remelted at 192 to 197°, identical in behaviour with *allo*gibberic acid hydrate. The identity was confirmed by comparison of the infra-red spectra. After drying gibberellin B at 100° C in vacuo a mixed melting point determination with anhydrous *allo*gibberic acid showed no depression.

DISCUSSION

Though the identity of *allo*gibberic acid and gibberellin B is certain there are discrepancies in reported biological activity that require explanation. Stowe and Yamaki (8) have noted two features of the biological activity of gibberellin B: 1) It stimulates shoot growth of rice seedlings, having approximately one tenth the activity of gibberellin A (Gibberellin A, as used by Yabuta et al, is now believed by Takahashi et al (9) to have been a mixture of two or more compounds). 2) Though it might have been suspected that such activity could best be accounted for by contamination with gibberellin A, this explanation must be rejected, because gibberellin B increased root growth, unlike gibberellin A which reduced it. Their conclusions were based on the data of Yabuta et al (10).

Since the alleged stimulation of root growth by gibberellin B is critical for this argument, the results of Yabuta et al (10) merit further examination. They presented data for growth of rice seedlings over periods of 8 and 15 days. These do indicate that root growth was slightly depressed by gibberellin A; this has been our experience with gibberellic acid on wheat seedlings (3) in experiments lasting one or two weeks, but in more critical tests over shorter periods (4) we have not found it to affect root growth in concentrations up to 10 μg/ml. Gibberellin B, on the other hand, had little if any effect on growth as judged by their 8-day data, but the 15-day data indicated marked stimulation of root growth by the two highest concentrations tested (3.5 and 7 μg/ml). It is doubtful whether these results can be accepted at their face value. The mean values presented were each based on measurements of 20 seedlings, growing in a very small container with no nutrients supplied. Such conditions are unsuitable for root growth studies, as is indeed shown by the wide variability within treatments indicated by the maximum and minimum values presented; indeed it is doubtful whether any of the effects on root growth could be considered significant. Moreover, Yabuta et al themselves concluded that gibberellin B had no effect on root growth, in distinction from gibberellin A which was slightly inhibitory.

If this point of view is accepted, it seems reasonable to conclude that our data on shoot growth for *allo*gibberic acid and those of Yabuta et al for gib-

berellin B can best be reconciled by assuming that their material was contaminated with the active gibberellin A. The results we have presented in this paper show the difficulty encountered in obtaining samples of *allo*gibberic acid uncontaminated with gibberellic acid, using chemical methods for preparation of the former from the latter. The gibberellin B used by Yabuta et al in their physiological studies (10) was extracted from culture filtrates containing more gibberellin A than gibberellin B, so that risks of contamination were even greater than in our case.

Our results indicate that *allo*gibberic acid does not promote shoot growth. *allo*Gibberic acid agrees in its properties with the published chemical and physical properties of gibberellin B (10, 11) and the hydrate was identical with a specimen of gibberellin B supplied by Professor Sumiki. Thus if our explanation of the results of Yabuta et al (10) is not accepted, it must be concluded that the gibberellin B used in their physiological experiments differed from that investigated chemically then and later, and differed from the gibberellin B supplied to us. This seems to us unlikely.

We have shown *allo*gibberic acid to inhibit growth of cress roots slightly at 10 μg/ml. This is not necessarily incompatible with the observations of Yabuta et al. They did not use gibberellin B at so high a concentration as this, and cress roots are in general more sensitive than those of rice or other cereals.

Perhaps the most important conclusion that can be drawn from these results, is that since *allo*gibberic acid differs from gibberellic acid only in ring A, this part of the molecule must be of importance in determining biological activity of the gibberellins.

Summary

*allo*Gibberic acid hydrate is shown to be chemically identical with the gibberellin B described by Yabuta et al (10). When rigorously purified it does not induce accelerated shoot growth of dwarf peas. It slightly inhibits the growth of cress roots, and this inhibition is not reversed by gibberellic acid. Earlier claims (10) that gibberellin B stimulates shoot growth are attributed to incomplete removal of gibberellin A. These results indicate that ring A of gibberellic acid is of importance in determining biological activity.

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BIOSYNTHESIS OF HORDENINE IN TISSUE HOMOGENATES OF PANICUM MILIACEUM L.[1,2]

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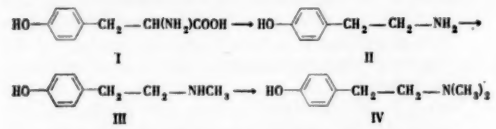
Interest in the mechanism of hordenine biosynthesis in plant tissue dates from 1937 when Raoul (17) proposed a series of reactions for the synthesis of the alkaloid. This scheme was based on the structural similarity of hordenine and the amino acid, tyrosine.



He postulated that tyrosine (I) is decarboxylated to tyramine (II) which through a stepwise methylation forms N-methyltyramine (III) and subsequently hordenine (IV). Attempts to verify this postulated reaction sequence have been partially successful.

Leete, Kirkwood and Marion (10) administered tyramine-2-C^{14} to sprouting barley and isolated radioactive hordenine and N-methyltyramine from the roots. The specific activity of the N-methyltyramine was about 10 times that of hordenine, and it was concluded that tyramine undergoes methylation in the barley root to form N-methyltyramine and then hordenine. Leete and Marion (11) then fed DL-tyrosine-2-C^{14} to sprouting barley and were able to isolate radioactive hordenine and N-methyltyramine from the roots of the plants. Degradation of these alkaloids showed that all of the activity was located in the 2-carbon of the side chain, indicating that tyrosine is a precursor of N-methyltyramine and hordenine. In a similar experiment Massicot and Marion (12) isolated radioactive hordenine from barley roots after feeding phenylalanine-2-C^{14} and considered this evidence for the conversion of phenylalanine to tyrosine in that plant. L-Methionine labeled with C^{14} in the methyl group was fed to sprouting barley by Matchett, Marion and Kirkwood (13). Radioactive hordenine was isolated and degraded. This showed that all of the radioactivity in the molecule was in the N-methyl groups. It was concluded that methionine serves as an efficient methyl donor for hordenine biosynthesis in barley seedlings.

In a recent work James and Butt (8) were able to verify in part the results of Marion et al. Tyramine and methionine were incubated with excised barley embryos under sterile conditions, and N-methyltyramine and hordenine were detected in the seedlings. Under the same conditions tyrosine and methionine failed to yield either N-methyltyramine or hordenine. These authors were also unable to detect tyrosine decarboxylase in barley roots.

It is evident that the different experimental methods of James and Butt, and Marion et al have yielded different results with respect to the role of tyrosine as a precursor of hordenine. A further evaluation of the role of this amino acid in hordenine biosynthesis might reconcile the differences.

Demaree and Tyler (5) investigated some aspects of hordenine metabolism in *Panicum miliaceum* L. (proso). Using a sensitive, quantitative, paper chromatographic technique they were unable to detect measurable quantities of the alkaloid in the roots of proso seedlings. Accumulation of hordenine in the proso shoot was found to be associated with a simultaneous disappearance of tyramine. This reciprocal association suggested a biosynthetic relation between the two compounds in proso, and the present study was undertaken to verify this relationship.

Hordenine appears in the roots of barley seedlings soon after germination. The alkaloid reaches a maximum concentration after approximately 12 days and then slowly disappears (18). It would be interesting to know whether the hordenine in proso disappears with increasing age as it does in barley. Experiments with intact proso seedlings were undertaken to determine if this occurred. Determination of the site of synthesis was also undertaken using separate homogenates of root and shoot tissue. Tyramine and methionine or methionine sulfoxide were incubated with homogenates of proso root and shoot tissue for periods of time ranging up to 72 hours. Tetracycline was added to the homogenates to prevent contamination with microorganisms. The addition of antibiotics to plant tissue cultures in which methylation reactions were being studied has been employed by Byerrum and Wing (4). Both methionine and methionine sulfoxide were tested as methyl donors since it had not been determined whether the enzymatic methylation system of proso was oxygen dependent (3). Adenosine triphosphate (ATP) is known to be involved in methylation reactions, but the compound is unstable in solution at 25° C and does not lend itself to the study of reactions requiring a prolonged incubation time. In this study ATP was not employed in homogenates incubated longer than 8 hours.

MATERIALS AND METHODS

TIME DISTRIBUTION OF HORDENINE: Proso seeds were soaked in water for one hour and then transferred to clay pots containing moist soil where they

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[2] The data in this paper were taken from a thesis presented by L. R. Brady to the faculty of the Graduate College of the University of Nebraska in partial fulfillment of the requirements for the degree of Master of Science in Pharmacognosy.

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were allowed to germinate and develop under normal greenhouse conditions. Groups of 8 plants were harvested after 10, 15, and 20 days.

The plants were removed from the soil and the roots washed with water. Shoots and roots were separated, dried in an oven at 45° C for 24 hours, then placed in a desiccator over anhydrous calcium chloride for at least 20 hours. The shoots (50 to 150 mg dry weight) were ground to a no. 20 powder in a Wiley laboratory mill. The roots (10 to 20 mg dry weight) were powdered directly in a mortar and transferred to 16 × 85 mm test tubes with 10-ml portions of alcohol. The powdered shoots were also placed in test tubes together with 10-ml portions of alcohol. All the tubes were stoppered and placed on a reciprocal shaking machine for two hours. The tubes were centrifuged, the extracts decanted into evaporating dishes, and the residues washed with two successive 2-ml portions of alcohol. The washings were combined with the original extracts. After the extracts were evaporated to dryness, the soluble portion of each residue was taken up in 1.0 ml of alcohol.

The shoot extracts of 10-day-old seedlings and the root extracts of 10-, 15-, and 20-day-old seedlings were tested chromatographically for hordenine. Quantities of the concentrated extracts representing 25 to 75 mg (dry weight) of shoot tissue and 5 to 10 mg (dry weight) of root tissue were chromatographed by the procedure of Leete, Kirkwood and Marion (10). One-inch strips of Whatman no. 1 filter paper were dipped in a pH 8 buffer solution composed of a mixture of 0.2 M disodium phosphate and 0.1 M citric acid. The strips were dried at room temperature and the extracts applied in successive 10 μl volumes. Each volume was allowed to dry before adding the next. Ascending formation of the strips was carried out for 24 hours with *n*-butanol saturated with water. Discrete and unequivocal separation of tyramine, N-methyltyramine and hordenine was obtained by this method. The compounds gave R_f values of 0.33, 0.44, and 0.83 respectively.

The large amount of chlorophyll present in the extracts of the 15- and 20-day-old plant shoots precluded the use of the *n*-butanol : water chromatographic system in their examination. These extracts were spotted on unbuffered strips of Whatman no. 1 filter paper and formed in an ascending direction with a 10 % ammonium sulfate solution, as suggested by Resplandy (19). This wash liquid did not give a discrete separation of hordenine and tyramine, both these compounds showing an average R_f 0.78. It did separate them from chlorophyll and made it possible to detect compounds giving a positive Pauly's reaction.

Control strips spotted with hordenine and tyramine, and mixed controls containing these compounds plus the plant extracts, were included in each chromatographic trial. At the conclusion of the formation period the strips were air dried and subjected to Pauly's reaction with sodium nitrite as described by Block et al (2). Demaree and Tyler reported that this reaction can detect as little as 2 μg of hordenine or tyramine. This reaction is not specific for these two compounds but reacts with many compounds containing a phenolic hydroxy group and with imidazoles. Used in combination with the chromatographic method it constitutes a reliable micro-procedure for the identification of hordenine and its precursors in proso (5). The quantities of the tissues examined in this experiment were sufficient to allow the detection of a minimum of 0.008 % of hordenine in the roots and 0.0007 % of that alkaloid in the shoots.

Biosynthetic Role of Proso Root: Proso seeds were germinated in moist air as described by Meyer and Anderson (14). The roots were excised from three- to five-day-old etiolated seedlings. Four-day-old seedlings were most commonly employed. The surface moisture was removed from the fresh roots by blotting with filter paper. They were then weighed and homogenized in a glass tissue grinder supported in an ice bath. The total homogenate was then divided and exact portions pipetted into 50-ml Erlenmeyer flasks. Each portion of homogenate represented approximately 0.5 g of fresh plant tissue. Solutions containing the postulated precursors were then added to the Erlenmeyer flasks. Tyramine was added as the hydrochloride equivalent to 2 mg of tyramine base (14.6×10^{-6} M). One mg of methionine or methionine sulfoxide was added to the homogenates (6.7×10^{-6} or 5.5×10^{-6} M respectively). A sufficient quantity of a broad spectrum antibiotic, usually tetracycline (Achromycin), to give a final concentration of 1 : 50,000 was also added to the homogenate samples when the incubation period was 24 hours or longer. The final volume of all homogenate samples was adjusted to 5.5 ml. Various media were used to prepare the homogenates and the solutions of the precursors. These included distilled water, M/15 Sorensen's buffer (pH 5.5, 6.5, or 7.5) (9) and Hoagland and Arnon's nutrient solution no. 1 (7).

No initial control was required since hordenine was not present in the root homogenates when the precursors were added. The control employed for the trials of the root homogenates was a sample in which the enzymes had been inactivated by heat. A sample of homogenate to which tyramine and either methionine or methionine sulfoxide had been added was autoclaved at a temperature of 121° C for 15 minutes. The 50-ml Erlenmeyer flasks containing the homogenates were stoppered with cotton and placed either on a reciprocal shaker at room temperature or in the dark in a constant-temperature cabinet at 26° C for an incubation period of 8, 12, 24, or 48 hours. More than 40 different combinations of the above variables were investigated.

At the conclusion of the incubation period, the samples were transferred quantitatively to glass centrifuge tubes. The supernatants obtained by centrifugation were decanted into small evaporating dishes. The residues were washed twice with 1- to 2-ml portions of distilled water and the washings were

combined with the initial supernatants. The combined liquids were evaporated to dryness in a stream of warm air, and each residue was taken up in 1.0 ml of alcohol. Thirty-50 μl quantities of the alcohol extracts were spotted on strips of Whatman no. 1 filter paper buffered at pH 8. The chromatographic strips were formed ascendingly with a *n*-butanol : water system in a Chromatocab as described previously. After the formation of the strips, they were subjected to Pauly's reaction and the results evaluated.

In spite of the care exercised and the antibiotics added, a few of the experimental homogenates were found to be contaminated with microorganisms at the end of the incubation period. Although the contaminated samples were routinely processed, the results obtained were not considered for further evaluation. The homogenates contaminated with bacteria exhibited a putrefactive odor and showed a decrease of tyramine and hordenine content if either was initially present. Fungal contamination was not generally a problem.

No hordenine could be detected in any of the root homogenate samples regardless of the identity of the added precursors, the nature of the suspending medium, or the length of the incubation period.

PROSO SHOOT HOMOGENATE CONTROLS: The presence of hordenine in homogenate samples of proso shoots required the utilization of three types of controls to evaluate effectively the influence of added precursors on the alkaloid content. The 1st type of control (initial control) was a homogenate which was prepared and processed immediately to indicate the amount of hordenine in the sample prior to the addition of the precursors. In the 2nd type of control the homogenate was autoclaved at 121° C for 15 minutes prior to incubation with appropriate addenda. All enzymes were thus inactivated. Hordenine was not decomposed by this treatment. A 3rd type of control consisted of a homogenate which was carried through the incubation period with viable enzyme systems but no added precursors. This measured the influence of the enzymes on the original hordenine content.

To test the validity and the relationship of the results obtained with each of these three types of controls, shoot homogenates were prepared and subjected to different combinations of the respective treatments (table I). The homogenates were prepared and processed in the same manner as the root homogenates, except Hoagland and Arnon's nutrient solution was used as the diluent for all of the homogenates, and all samples were placed in the constant-temperature cabinet for the selected incubation period. Approximately 1 g of fresh tissue was employed for each homogenate. Extracts of the shoot homogenates were prepared and chromatographed in the same manner as the root homogenates. The chromatograms were evaluated quantitatively by the procedure of Demaree and Tyler (5).

This is a method developed especially for the separation and micro-determination of hordenine in proso. Chromatograms of hordenine and its precursors are prepared essentially as described by Leete et al (10). The compounds are rendered visible by Pauly's reaction, and the maximum color density of the hordenine spots are measured with a Photovolt Electronic Densitometer, Model 525, fitted with a 1×15 mm slit and a 465 mμ narrow-band gelatin filter. The amount of hordenine on the chromatogram is determined from a standard curve showing the relationship between maximum color density and hordenine concentration.

A sufficient number of chromatograms were employed to allow adequate statistical evaluation. All of the values are reported as the color density per 30-μl volume of an alcoholic extract. Occasionally it was necessary to spot 20-μl volumes of the more concentrated extracts so that the observed densities would fall within the sensitive range of the densitometer. In such cases the color densities of 30-μl volumes were calculated from the established relationship between the amount of hordenine and the color density (5). The mean and standard error of the mean were calculated for each group of density readings according to the formulas $\bar{x} = \Sigma(x)/N$ and $s_{\bar{x}} = s/\sqrt{N}$ respectively (1, 15). When the means of two treatments were to be compared, the variance of the readings of each treatment was calculated by the formula $s^2 = \frac{\Sigma(x^2) - (\Sigma x)^2/N}{N-1}$ as given by Bliss and Calhoun and the variance ratio determined to establish their compatibility. After it had been established that the groups of readings were compatible, the "t" test, $t = \frac{\bar{x} - \bar{y}}{s}\sqrt{\frac{nm}{n-m}}$, was applied as described by Youden (20) and the probability level obtained from a standard statistical table (6).

In each pair of samples the mean values of the

TABLE I

RESULTS OF ANALYSES FOR HORDENINE IN PROSO SHOOT HOMOGENATE CONTROLS

TYPE OF CONTROL	PRECURSORS ADDED	INCUBATION (HRS)	NO. OF STRIPS	DENSITY READING (30 μL) MEAN ± S.E.
Autoclaved	Tyr + meth sul *	60	22	0.338 ± 0.012
Initial	None	0	16	0.339 ± 0.015
Autoclaved	Tyr + meth sul	60	17	0.307 ± 0.013
Initial	None	0	11	0.339 ± 0.010
Autoclaved	Tyr + meth	48	18	0.322 ± 0.010
Initial	None	0	10	0.312 ± 0.010
Viable enzyme	None	48	15	0.377 ± 0.009
Autoclaved	None	48	13	0.383 ± 0.010

* Tyr = tyramine; meth sul = methionine sulfoxide; meth = methionine.

density readings were similar (table I). This indicated that the initial control, the autoclaved control, and the viable enzyme control were equivalent and could be employed interchangeably as an accurate measure of hordenine in homogenates lacking added precursors.

Biosynthetic Role of Proso Shoot: To determine the influence of certain precursors on the amount of hordenine in a shoot homogenate, tyramine and methionine or methionine sulfoxide were added to the sample which was allowed to incubate in the constant-temperature cabinet for the specified period of time (table II). A similar homogenate sample was also processed in one of the ways described previously to serve as a control. The influence of the added precursors was established by comparing the amount of hordenine in the control with that in the sample containing the precursors. The samples were processed and evaluated in the manner described for the shoot homogenate controls.

The results indicated that tyramine, in the presence of methionine or methionine sulfoxide, was converted to hordenine by the enzymes in proso shoot homogenates.

Results and Discussion

Neither hordenine nor tyramine could be detected in the root extracts of the intact proso seedlings. Hordenine was present in the shoots of 10-day-old plants, but tyramine was not detected. A minute quantity of hordenine was detected chromatographically in the shoots of 15-day-old plants, but it could not be found in the shoots of 20-day-old plants. Therefore, hordenine was shown to disappear from the shoots of proso seedlings between 15 and 20 days after germination.

In this respect, the pattern of hordenine accumulation and disappearance in the proso shoot is similar to that of hordenine in the barley root. It is of considerable interest to note the related metabolic patterns of the same alkaloid in different morphological parts of the two plants.

No hordenine was detected in any of the root homogenates which had been subjected to the experimental conditions.

Chromatograms spotted with an extract of root homogenates which had been incubated 8 hours showed only one spot giving a positive Pauly's reaction. This spot corresponded to tyramine which had been added initially to the homogenates. The chromatographic evaluation of the extracts of the root samples which had been subjected to a minimum 12-hour incubation period and had not been treated to serve as controls showed two spots in addition to the tyramine. These spots had average R_f values of 0.93 and 0.21, and it was concluded that neither of them was hordenine or N-methyltyramine. No attempt was made to characterize them further.

The results obtained with shoot homogenate samples are summarized in table II. When the "t" test was applied to the mean color-density readings of each treated sample and its corresponding control, the probability levels which were obtained indicated that the observed increases in the maximum color densities of the hordenine spots of the treated homogenate samples were statistically significant for a biological process.

Under the conditions employed in this investigation it was not possible to obtain any evidence to support the hypothesis that hordenine is formed in the roots of proso seedlings and immediately transported to the shoots. Furthermore, the evidence obtained by the application of the tissue homogenate technique showed that hordenine was formed in shoot homogenate samples from tyramine and a methyl donor. Evidence for the exact mechanism of the methylation reaction was not conclusive, but both methionine and methionine sulfoxide were capable of serving as methyl donors. The evidence that hordenine is synthesized from these added precursors in the shoot homogenate may not be applied to the intact plant without some reservation; however, it may be considered as presumptive evidence for such a biosynthetic pathway in the shoot of the intact seedlings. The suggested function of proso shoot in alkaloid biosynthesis is interesting in view of the limited

Table II

Results of Analyses for Hordenine in Proso Shoot Homogenate Samples

Treatment	Incubation time (hrs)	No. of strips	Density reading (30 μl) Mean ± S.E.	Probability level
Tyr + meth sul *	72	7	0.426 ± 0.014	< 0.1 %
Viable enzyme control	72	12	0.254 ± 0.013	
Tyr + meth	48	13	0.403 ± 0.014 **	0.1 to 1.0 %
Tyr + meth sul	48	23	0.370 ± 0.008 **	2.0 to 5.0 %
Initial control	0	11	0.339 ± 0.010	
Tyr + meth sul	48	23	0.423 ± 0.007 **	< 0.1 %
Autoclaved control (Tyr + meth added)	48	18	0.322 ± 0.010	

* Tyr = tyramine; meth sul = methionine sulfoxide; meth = methionine.
** Density readings were calculated from the respective readings for 20-μl volumes.

number of plants in which the shoot synthesis of alkaloids has been definitely established (16).

Faint spots with R_f values corresponding to N-methyltyramine were observed on chromatograms spotted with extracts of shoot homogenates. On the basis of the positive Pauly's reaction and the R_f value with the chromatographic system employed, it was concluded that N-methyltyramine was present in the shoots of the proso seedlings. The low color density of the spot indicated that a relatively small amount of this substance was present in comparison to the amount of hordenine in the shoots. The minute amount of N-methyltyramine in the proso shoots probably accounts for the fact that it was not detected in the preliminary study carried out by Demaree and Tyler.

Summary

1. Hordenine disappeared from the shoots of intact seedlings of *Panicum miliaceum* between 15 and 20 days following germination.

2. Tissue homogenates were used to study the role of proso roots and shoots in hordenine biosynthesis. The proso root could not be shown to function in hordenine biosynthesis under the conditions employed, but shoot homogenates were found to be capable of forming the alkaloid when tyramine and a methyl donor were present. Methionine and methionine sulfoxide served as methyl donors of nearly equal efficiency.

3. The results obtained from studies of proso homogenate samples may be considered as presumptive evidence for the biosynthesis of hordenine from similar precursors in the shoot of the intact proso seedling.

4. N-methyltyramine was detected chromatographically in proso shoots.

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THE CHEMICAL NATURE OF SILICA IN PLANTS[1]

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Silica has long been known to be present in plants. Richardson (6) reported its abundance in the aerial parts of plants of the Equisetum genus and many Gramineae, constituting 50 to 70 % of the ash. He also stated that of all elements found in plants, silicon showed the greatest variation between plant parts, plants, and species of plants. Silicon usually occurs in plants in the form of its oxide, SiO_2, commonly called silica.

Although the presence of silica has been established its chemical form in higher plants has not been reported. This study was conducted primarily to provide such information. Since opal and α quartz are produced and exist as minerals at temperatures favorable to plant growth one might expect that silica in plants would be one or the other or both.

According to Iler (2) the solubility of amorphous silica at ordinary temperatures is most commonly reported to be about 0.010 to 0.015 %. Esau (1) stated that some plants absorb more silica than they need and inasmuch as it cannot be excreted, it is deposited in the tissues. In general, the more water absorbed by a plant, the greater the amount of silica deposited. Silica is deposited mostly in cell walls, but sometimes as bodies in the lumen of the cell. The Gramineae is the best known group depositing silica; the deposition takes place in both cell walls and cell lumina. Silica is not usually deposited in underground parts.

In this work the silica was characterized by chemical analysis, petrographic microscope studies, and x-ray diffraction analysis. The petrographic microscope was used for the initial identification of the type of silica present. The optical characteristics of opal and quartz are quite different so that this method of study is readily used to differentiate between them. X-ray diffraction studies were conducted to supplement the information gained by the petrographic microscope and to show the presence of minor constituents. The x-ray diffraction pattern of α quartz has been determined by Swanson and Fuyat (7) and x-ray studies of opal have been made by Swineford and Franks (8).

This study has also established: (A) unreported types of silica deposition and (B) tissues other than the epidermis as sites of silica deposition. Spodograms were used for this purpose as they are the best method for studying the sites and depositional pattern of silica in situ. Spodograms are made by mounting ash material on microscope slides. The process was developed by Uber (9) and used by Molisch (3) and Ohki (4) to get distinct, transparent skeletal deposits. Usually, transverse or longitudinal sections of plant tissues are used in the preparation of spodograms. For thin parts such as leaves, the entire tissue has been used by Ponnaiya (5). Ohki (4) studied in detail the spodograms of leaf blades of the Japanese Bambusaceae, covering 6 genera and various species. He found that the pattern of silica deposition was constant and distinct for each species. Ponnaiya (5) modified the technique for preparing spodograms. The material to be studied was placed flat between microscope slides. The material was then ashed in a muffle furnace at between 450 and 500° C. The ash was prepared for study by removing the upper slide, adding Canada balsam directly to the ash mass, and covering with a cover glass.

Ponnaiya (5) working on sorghum, found silica to be present in the epidermises of the leaf and the leaf sheath, in the node and internode of stems, in the glume and awn of floral parts, and in traces on the surface of the grain. He described two types of silica deposition in sorghum leaves: (a) dumb-bell shaped structures occurring in regular rows about the veins, and (b) irregularly shaped structures occurring scattered between the regular rows of the dumb-bell shaped units.

MATERIALS

Since silica is deposited both in monocotyledonous and dicotyledonous plants, common representatives in both groups were studied. Leaves and leaf sheaths of three common crops of the family Gramineae: Concho variety of wheat (*Triticum vulgare*), Westland variety of sorghum (*Sorghum subglabrascens*), and an inbred line, Kansas 201, of corn (*Zea mays*) were used. Bamboo cane was also studied since it has been reported to have heavy depositions of silica.

Among dicotyledonous plants, sunflower (*Helianthus annuus*) and lantana (*Lantana camara* Linn.) were studied. Silica deposition has been reported in sunflower but not in lantana. Both leaves and stems of these species were studied.

METHODS

Depositional patterns of silica and shapes of individual silica particles were studied by the spodogram technique described by Ponnaiya (5). A few crystals of phenol were added as a dehydrating agent to the Canada balsam to prevent the appearance of bubbles. The silica particles were measured by means of an ocular micrometer. Their sizes are given in table I.

Most of the samples used in the x-ray and petrographic microscope studies were made by completely ashing oven-dried plant material in the temperature range of 700 to 900° C. An atmosphere of pure oxygen was used to remove the carbon from both sunflower and lantana ash. In sorghum, only the epi-

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TABLE I

SIZE OF SILICA PARTICLES IN PLANTS

PLANT	PART MEASURED	MEASUREMENTS IN MILLIMETERS
Sorghum	1. Silicified serrated leaf edge	
	A. tooth tip to tooth tip	0.129 to 0.215
	B. diameter of tooth at base	0.089 to 0.136
	2. Dumb-bell shaped particles	0.0135 × 0.0207
	3. Irregular shaped particles	0.007 × 0.0152
	4. Rectangular particles in leaf blade (subepidermal)	
	A. width of rows of particles	0.0198 to 0.0316
	B. length of particles in rows	0.017 to 0.0730
Wheat	Oval particles in wheat leaf sheath	0.017 × 0.0153
Corn	Rectangular particles in leaf blade	0.115 × 0.0216
Bamboo	Rod-like particles in the nodes	
	A. Width	0.0126
	B. Length	0.414 to 0.566
Lantana	1. Silicified cells at base of trichomes	0.0496 × 0.0712
	2. Maximum length of trichomes	0.462
	3. Maximum width of trichomes	0.0531
Sunflower	Diameter of largest particles	0.064

dermis of the leaf sheath was used. The ash was repeatedly treated with hydrochloric acid to remove mineral impurities and the silica then dried at 110° C.

In determining percentage ash and percentage silica in the ash of plants, the same method was followed except that the silica was ignited and determined gravimetrically. The silica was checked with hydrofluoric acid to insure it being pure SiO_2. Ash and silica percentages are given in table II.

A second means of preparing a sorghum sample for petrographic microscope and x-ray studies was to macerate the leaf sheath epidermis with water in a Waring blendor. The fibrous material was removed and the finer material was dried at 110° C. This material had a high silica content. Samples of sorghum and lantana were dried at 110° C, ground in a mortar and pestle, and the finely ground substances studied by both methods.

TABLE II

SILICA IN PARTS OF PLANTS

PLANT	PART OF PLANT	% ASH	% SiO_2 IN ASH
Westland sorghum	Leaf sheath epidermis	12.55	88.70
Concho wheat	Whole leaf sheath	10.48	90.56
Corn (Kansas-201)	Whole leaf blade	12.15	64.32
Bamboo	Nodes (inner portion)	1.49	57.40
Lantana	Composite sample (leaf and stem)	11.24	23.28
Sunflower	Composite sample (leaf and stem)	11.53	25.32

X-ray diffraction patterns of the silica samples were made on a North American Phillips diffractometer utilizing nickel filtered copper radiation obtained with a current setting of 40 kilovolts and 20 milliamperes. A one degree slit system and a goniometer scanning speed of one degree two-theta per minute was employed in conjunction with a chart scale factor of 400 and a time constant of four.

RESULTS

Petrographic microscope studies of the silica from the ash of sorghum, wheat, corn, sunflower, and bamboo show it to be clear, colorless, and isotropic with an index of refraction of 1.45. These properties are typical of the mineral opal. Many of the larger opal particles showed a cellular structure that was probably retained from the shape of the cell in which they were formed. Minute pieces of carbon were observed in the recessions of many of the opal particles.

Similar studies of the silica from lantana indicated that part of it was typical of the opal observed in the other plants with an index of refraction of 1.45. The remainder of the silica in lantana was found to be clear, colorless and anisotropic with all the optical properties characteristic of α quartz. It was uniaxial positive with indices of refraction of $\omega = 1.54$ and $\epsilon = 1.55$.

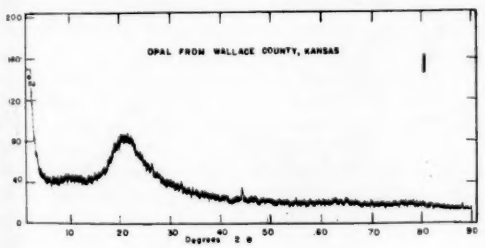
Samples of the silica from sorghum and lantana prepared by drying at 110° C and then grinding with a mortar and pestle were also studied to determine if the material was changed by laboratory treatments. The silica in these samples was the same as that noted in the ash. Traces of cristobalite were noted in some of the ashed samples that were heated to 900° C.

X-ray diffraction patterns for opal from Wallace County, Kansas and silica from the sorghum plant are given in figures 1 and 2 respectively. The two patterns are identical which indicates that the silica in sorghum is opal. There were no x-ray diffraction peaks of the crystalline forms of silica such as quartz or cristobalite in the patterns, therefore those varieties of silica must be absent.

Swineford and Franks (8) recognized two major types of opal in the Ogallala Formation in Kansas, one of which they classify as a diatom or biogenetic type and a second which they refer to as a low-cristobalite-tridymite type. Their biogenetic or diatom type of opal presented x-ray diffraction patterns which exhibited only two broad swells or diffraction bands; one at nine degrees two-theta and the other between 18 and 26 degrees. The opal patterns shown in figures 1 and 2 are typical of the x-ray patterns presented by their biogenetic type of opal except for the small peak at 44.25 degrees. That peak is probably due to a small amount of carbon which has a major peak at that position.

X-ray studies of silica from lantana, figure 3, show the broad diffraction bands attributed to opal plus all the sharp peaks characteristic of α quartz. Slow scanning with x-rays indicated traces of cristobalite in a few of the samples of both lantana and sorgrum that were ashed at temperatures near 900° C. This was probably due to heating because no cristobalite lines were noted in the x-ray traces of the majority of the ashed samples and none were observed in any of the samples that were prepared by maceration and grinding instead of ashing. This indicates that care must be taken in preparing plant tissues for study by the ashing process. The material should be heated at as low a temperature as possible and for no longer period than necessary in order to prevent the conversion of silica to some form other than that which was actually present in the plant. The x-ray diffraction patterns of the samples of sorghum and lantana prepared by grinding and maceration were the same as those for silica obtained by the low temperature ashing-hydrochloric acid treatment, showing that the chemical form of silica was not altered by this treatment. The cellulose that was left in these samples did not mask the x-ray lines of opal or quartz.

Leaf blades and leaf sheaths of sorghum, corn, and wheat were studied. Spodograms revealed that silica was deposited only on the abaxial surface of the leaf sheath but was deposited on both surfaces of the leaf blade as described by Ponnaiya (5) for sorghum. Silica was also found deposited on all walls of sorghum epidermal cells, figure 4. Ponnaiya (5) observed that there were regularly and irregularly shaped silica particles in the abaxial epidermis of the sheath and both epidermises of the leaf blade of sorghum. In this study, the same was found for corn.



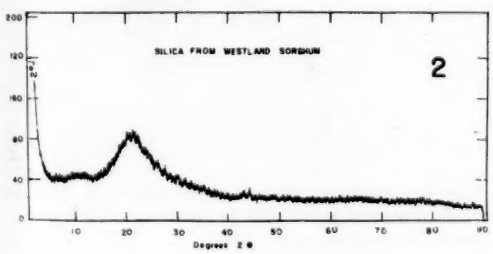


FIG. 1. X-ray diffraction pattern of opal.

FIG. 2. X-ray diffraction pattern of silica from Westland sorghum.

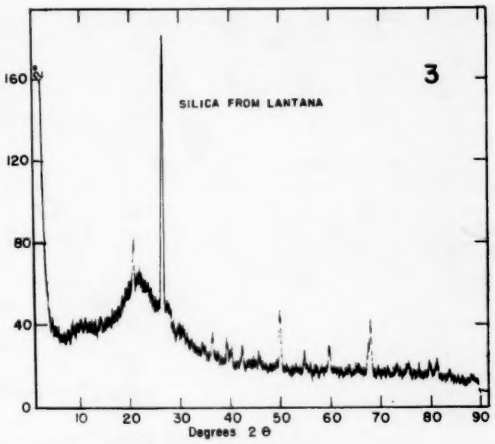


FIG. 3. X-ray diffraction pattern of silica from a composite sample of the leaf and stem of lantana.

The regularly shaped silica particles in the epidermises of sorghum and corn were dumb-bell shaped in outline, whereas they were ellipsoidal in wheat epidermis, figure 5.

Two unreported types of silica depositions were found in leaves. The leaf margins are silicified in corn, wheat and sorghum. The margin is also serrated in sorghum, figure 6. The topmost fully developed leaf of a month-old sorghum plant had a well-formed, serrated, margin which was highly silicified.

The second unreported type of silica deposition occurred only in old mature leaves of mature plants. The particles were rectangular in shape and occurred in long rows in the sub-epidermal layers of leaf blades of corn and sorghum, immediately below the area of the epidermis containing the irregularly shaped silica particles, figures 7 and 8.

Considerable silica deposition apparently takes place on the sorghum leaf margin before much is deposited in the leaf blade proper. This conclusion is substantiated by figures 8 and 9, which show respectively the silica deposition in a four-month-old and in a one-month-old leaf blade of sorghum. Both blades had well developed and silicified leaf margins but the younger leaf had very little silica deposition in the blade proper. Silica was also found deposited in the epidermis of nodes and internodes of corn, sorghum and wheat.

In bamboo cane, nodes and internodes but not leaves were studied. The general epidermal pattern was similar to those of wheat, corn and sorghum. When the entire nodal material was ashed and mounted, a long rod type of silica particle was found, figure 10. Using a series of spodograms these were found to be present in the nodes near the center of the stem.

In sunflower and lantana, both leaf blade and stem were studied by means of spodograms. Unlike

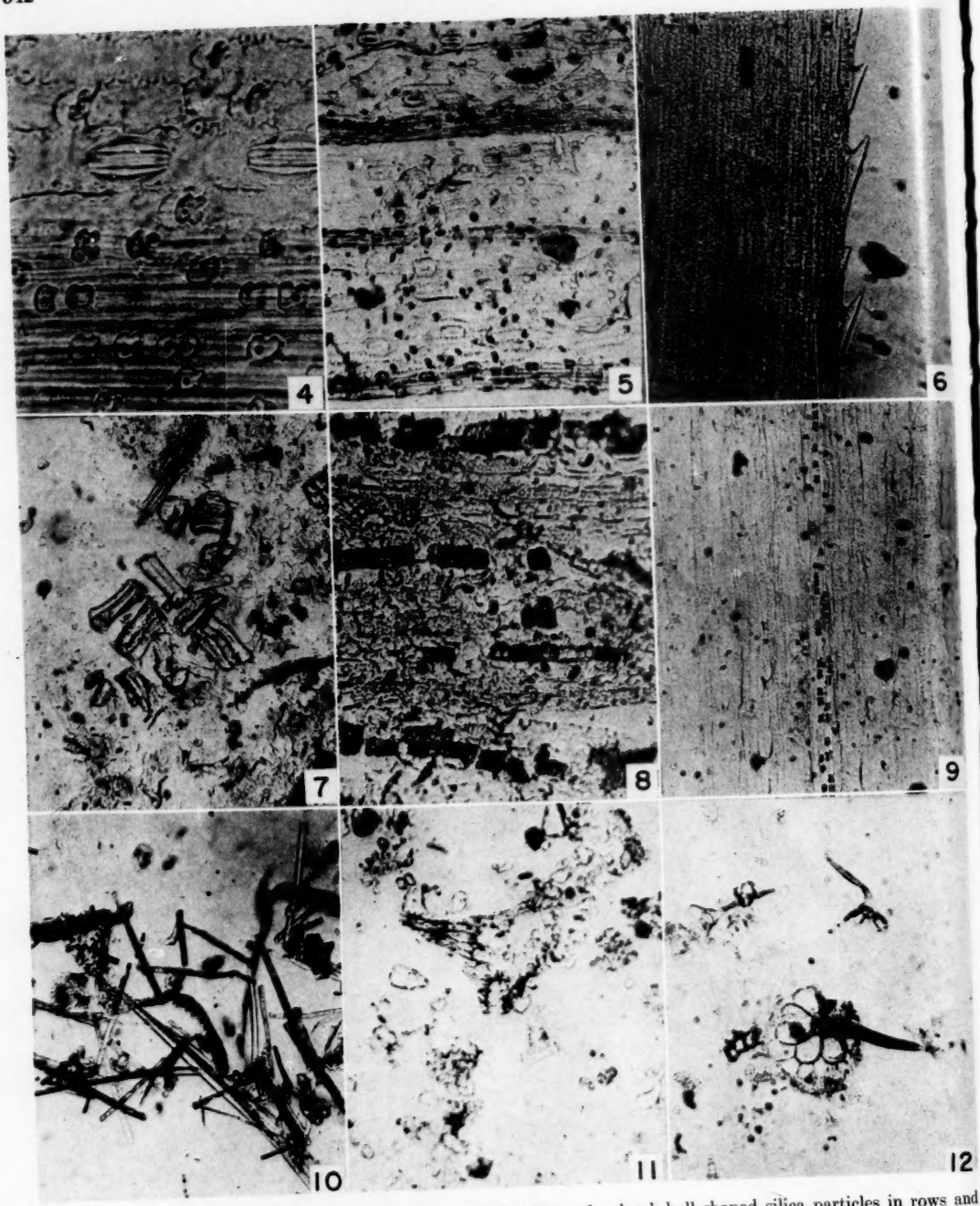


FIG. 4. Spodogram of sorghum leaf sheath epidermis. Note the dumb-bell shaped silica particles in rows and the scattered irregularly shaped particles. The silicification of cell walls and guard cells is clearly seen. 300×.

FIG. 5. Spodogram of wheat leaf sheath. Note the numerous elliptical particles. 80×.

FIG 6. Spodogram of the margin of a young sorghum leaf blade. Extensive silica deposition has occurred on the serrated margin. 80×.

FIG. 7. Spodogram showing the rectangular silica particles in the subepidermal layer of mature corn leaf blade. 80×.

the four monocotyledonous plants, the cell walls were not silicified. In sunflower epidermis a large number of irregular shaped silica particles were found, figure 11. The hairs on this plant were not silicified although figure 11 shows there is a tendency for silica deposition at their bases. In lantana only the prickle-like trichomes and the walls of epidermal cells at their bases were silicified, figure 12. These protuberances were usually hollow indicating that silica deposition was confined primarily to their walls.

Discussion

Determination of the x-ray diffraction pattern of silica from a plant part is a practical way of identifying the type of silica deposited in the plant. X-ray diffraction patterns for each form of silica are specific and no other compounds have the same patterns. Furthermore, ashing at low temperatures followed by treatment with hydrochloric acid does not appear to change its chemical form. In the sorghum leaf sheath, the x-ray pattern was made directly on dried plant tissue in which the silica content had been concentrated by physical means. Cellulose did not affect the x-ray diffraction pattern of silica.

Opal was the form of silica deposited in all plants studied, except lantana where α quartz was also present, figure 3. Further studies with other varieties of plants containing silica should confirm whether the deposition of α quartz is a specific characteristic feature of the species or whether it is a positional effect of the trichomes which protrude from the surface.

In the four monocotyledonous plants studied the cell walls of the epidermis also became silicified and each gave a characteristic depositional pattern (figs 4 to 9). In both dicotyledonous plants studied, no deposition was found in cell walls and hence no depositional pattern was observed. This may be a differential characteristic between monocotyledonous and dicotyledonous plants and needs further investigation.

Although sunflower has a considerable deposit of silica, table II, and has numerous epidermal hairs, these hairs do not show silica deposition, figure 11. In lantana, however, only the prickle-like trichomes are silicified, figure 12, indicating that each plant species may have an individual way of depositing silica.

Since the silica in sorghum, wheat, corn and sunflower is opal (amorphous) it is probable that the characteristic shape of the particles is determined by the cavities they come to occupy in the various tissues.

Summary

1. By means of petrographic microscope and x-ray diffraction studies, the silica deposited in sorghum, wheat, corn, sunflower, and bamboo has been shown to be opal.

2. X-ray and microscope studies show that the silica deposited in lantana is composed of both opal and α quartz.

3. By means of spodograms and microscopic examinations, the actual patterns and particle sizes of the silica deposits in these plants has been determined. A type of subepidermal deposition apparently not reported in the literature was observed in the leaf blades of sorghum and corn, between the rows of the dumb-bell shaped particles of silica. These particles occur in rows and are rectangular in shape.

The leaf blade margins of sorghum, wheat and corn are silicified. In sorghum the leaf margin is serrated and the serration becomes more distinct with age. Marked deposition of silica occurs on the serrated margins of sorghum leaves before much deposition occurs in the blade proper.

In addition to the epidermal deposition of silica in the cane of bamboo, long silica rods were observed in the internal tissue at the node.

4. In lantana and sunflower, the cell walls were not silicified and the spodograms did not show a pattern other than the shape of the individual particles. The particulate deposits in sunflower are irregular in shape. In lantana the prickle-shaped trichomes are silicified. As far as the authors are aware this is the first record of silica deposition in lantana.

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Fig. 8. Spodogram showing the sub-epidermal deposition of rectangular silica particles in a mature sorghum leaf blade. 80×.

Fig. 9. Spodogram of a month-old sorghum leaf blade. Note the scanty deposition of silica. 80×.

Fig. 10. Rod-like silica particles from the internal tissue of the node of bamboo cane. 80×.

Fig. 11. Irregular shaped silica particles from sunflower epidermis. 80×.

Fig. 12. Spodogram of lantana. Note the silicified trichomes with basal silicified cells. 80×.

COMPOSITIONAL FACTORS ASSOCIATED WITH THE GROWTH RESPONSES OF YOUNG COTTON PLANTS TO GIBBERELLIC ACID[1]

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In preliminary experiments (4) it was found that the growth of both normal and mutant cottons, like that of more than 80 other species (6), is influenced by gibberellic acid (GA_3). It is the purpose of this paper to report the effects of serial amounts of GA_3 on the growth and major chemical constituents of young cotton plants.

Plants for this experiment (var. Empire WR) were grown November 1 to December 6, 1956 in the greenhouse at 85° C in three-gallon jars of manured Houston black clay. When the plants were 15 days old (days after planting) and contained one true leaf, 0.01 ml of an aqueous solution of the sodium salt of GA_3,[2] containing 0.0, 0.1, 1.0, 10, or 100 μg of GA_3, was applied to the 1st true leaf of each plant. Each treatment comprised 8 plants. The applications to the apical leaf were repeated at two-day intervals for a total of 20 days. The last application was made two days prior to plant harvest.

At 35 days (preflowering stage) the tops from 7 plants in each treatment were separated into leaf blades and stems plus petioles and samples of the two tissues were analyzed for carbohydrates, lignin, nitrogen, and total ash constituents by methods previously reported (2, 3). Plants from the 0.1 μg series, for the reason stated in the following paragraph, were not analyzed.

GROWTH RESPONSES: With exception of the 1.0 μg dose, which was ineffective, there resulted stepwise increases in plant height with increasing GA_3 levels. The number of main-stalk nodes, however, was relatively unaffected (table I, fig 1). The 10 and 100 μg doses appeared to increase the fresh and dry weights of the stems plus petioles but had little or no consistent effect on the leaves (tables I, II). Root weight was not changed. The 1000 μg level, however, markedly reduced leaf weight, together with that of the entire plant, and caused yellowing. The latter resembled nitrogen deficiency symptoms in that the lower leaves exhibited the most severe chlorosis. In addition, the highest dose reduced the diameter of the main-stem, but not the dry weight of the stems plus petioles, and produced a condition similar to lodging in small grains.

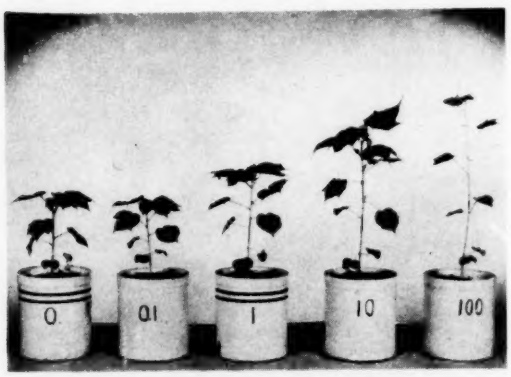

FIG. 1. Cotton plants at 35 days following treatment with 0, 0.1, 1, 10, and 100 μg of gibberellic acid (GA_3) at 2-day intervals for a total of 20 days.

TABLE I

FRESH WEIGHT, HEIGHT AND NUMBER OF MAIN-STEM NODES OF 35-DAY COTTON PLANTS AFTER TREATMENT WITH SERIAL AMOUNTS OF GIBBERELLIC ACID (GA_3) *

GA_3 per plant		Fresh weight of tops *			Height	Main stem nodes
Amount per application	Total dose	Leaves	Stems plus petioles	Total		
μg	μg	g	g	g	cm	*number*
0.0	0	7.8	4.4	12.2	23.6	8.2
0.1	1	7.7	4.3	12.0	23.2	8.2
1.0	10	8.4	4.9	13.3	27.3	8.0
10.0	100	7.6	7.5	15.1	44.0	8.5
100.0	1000	2.7	4.3	7.0	47.2	7.5

* Average values per plant.

** There was no significant effect of GA_3 on growth of roots.

LABILE CARBOHYDRATES: In the leaves the concentration of sugars increased as the level of GA_3 was increased but changes in starch and hemicellulose were not as consistent (table II). The percentages of sugars in the stems plus petioles, in contrast to that of the leaves, decreased through the 10 to 100 μg levels of GA_3 but increased at the 1000 μg level. At the same time the behavior of the starch fractions was even more erratic. Stems plus petiole hemicellulose, on the other hand, consistently increased in concentration as the supply of GA_3 was increased.

CELLULOSE AND LIGNIN: In the stems plus petioles, but not in the leaves, these cell wall constituents increased stepwise through the full series of GA_3 supply levels. In the former tissue, cellulose plus

[1] Received March 27, 1958.

[2] From lot No. 57R65 supplied by Merck and Co., Inc.

TABLE II

EFFECT OF GIBBERELLIC ACID (GA_3) ON THE DRY WEIGHT AND CHEMICAL COMPOSITION OF YOUNG COTTON PLANTS *

GA_3 per plant		Dry weight per plant	Total sugars	Starch	Hemi-cellu-lose	Cellu-lose	Lignin	Nitrogen			Ash
Amount per application **	Total for 20-day period							Soluble	Protein	Total	
μg	μg	g	%	%	%	%	%	%	%	%	%
					Leaves						
0	0	0.97	0.41	3.27	4.46	9.92	3.86	0.85	3.68	4.53	18.65
1	10	1.04	0.56	2.35	5.80	9.48	3.86	0.80	3.73	4.53	19.18
10	100	0.94	0.80	3.40	3.29	10.50	4.00	1.13	3.33	4.46	20.24
100	1000	0.36	2.22	4.79	6.79	10.72	4.20	0.96	2.53	3.49	18.42
					Stems plus petioles						
0	0	0.45	0.91	1.78	11.46	40.51	6.56	1.46	0.98	2.44	17.74
1	10	0.52	0.58	1.46	11.95	42.52	7.05	1.44	1.04	2.48	16.64
10	100	0.68	0.38	1.78	12.49	48.24	7.76	1.08	1.17	2.25	15.48
100	1000	0.49	1.84	1.04	14.13	56.50	9.19	0.93	0.67	1.60	10.49

* Percentages on dry weight basis.
** Plants of the 0.1 μg rate were not analyzed as they showed no observable growth response.

lignin made up 47 % (0 μg GA_3) to 65 % (1000 μg GA_3) of the dry weight.

NITROGENOUS AND ASH CONSTITUENTS: Marked differences in the concentrations of soluble-N, protein-N, and total-N, in either the leaf blades or stems plus petioles, were limited to the plants receiving the highest dose of GA_3. The leaves of these plants contained less protein-N and total-N than the untreated plants while the stems plus petioles contained less of all three fractions. However, until the inhibitory level was reached, total nitrogen (table III) in the stems plus petioles, as did total ash, increased until the inhibitory level (1000 μg) was reached.

DISCUSSION

The results of this study show that at the growth promoting levels, the effect of GA_3 was largely confined to the stems and petioles of the cotton plants. It consisted of increases both in stem elongation and dry weights. Growth inhibition was observed only at the highest level (1000 μg) of GA_3 used and was evidenced by a decrease in the dry weights of the leaves, but not of the stems plus petioles.

TABLE III

EFFECT OF GIBBERELLIC ACID (GA_3) ON TOTAL NITROGEN AND ASH CONSTITUENTS OF STEMS AND PETIOLES *

Total GA_3 per plant	Nitrogen	Ash
μg	g	g
0	0.011	0.842
10	0.013	0.865
100	0.015	1.050
1000	0.008	0.514

* Results as grams per plant.

Although the effects of the various levels of GA_3 on the more labile carbohydrate fractions, sugars and starch, showed inconsistent fluctuation, the sum of all the carbohydrate fractions, alone and in combination with lignin, shows remarkable consistency with treatment level and with tissue (table IV). As shown, the effect of GA_3 on total carbohydrates alone or including lignin, at less than inhibitory levels, is limited to the stems and petioles; the leaves are essentially unaffected until the 1000 μg level is reached. Thus the conclusion is indicated that the effect of GA_3 at doses promoting weight increases in this experiment is limited to the stems and petioles which are not only elongated by GA_3 but increased in dry weight and in the percent of fixed carbon involving carbohydrates and lignin. Additionally, it is noteworthy that this trend was maintained in the stems and petioles even at levels of GA_3 which were inhibitory to the weight gain of the plant as a whole. At the inhibitory level, however, the increase in total carbohydrates and lignin may have been at the ex-

TABLE IV

TOTAL CARBOHYDRATES (CH_2O) AND LIGNIN OF LEAVES AND STEMS PLUS PETIOLES *

Total GA_3 per plant	Leaves		Stems plus petioles	
	Total CH_2O	CH_2O plus lignin	Total CH_2O	CH_2O plus lignin
μg	%	%	%	%
0	18.06	21.92	54.66	61.22
10	18.19	22.05	56.51	63.56
100	17.99	21.99	62.89	70.65
1000	24.52	28.72	73.51	82.70

* Expressed as percentages of dry weight—taken from table II.

pense of ash and nitrogen, which decreased in total amount and concentration.

The findings of this study, with minor exceptions, agree remarkably well with those of Brian et al (1) on wheat and peas and Hayashi et al (5) on rice. However, the promotion of elongation by gibberellin is not always accompanied by a corresponding increase in dry weight or change in the major plant constituents. Wittwer and colleagues (7), for example, noted accelerated growth of Kentucky bluegrass by gibberellin without any significant alteration of the dry matter or mineral composition of the grass. Similarly, Yabuta et al (8, 9) reported (a) yield, total nitrogen, and crude fiber of tea leaves and (b) dry weight, ash, and nitrogen content of rice seedlings to be unchanged by gibberellin.

Mild to severe chlorosis often follows the treatment of young plants with gibberellin and in some instances, but not all, is corrected by the application of mineral nutrients, particularly nitrogen. In the instance of cotton, with an adequate external supply of nutrients, only the highest dose of GA_3 produced chlorosis which was associated with low ash and nitrogen (protein-N principally) contents of both the leaf and stem plus petiole fractions. This suggests interference with nitrogen metabolism at inhibitory levels of GA_3.

Summary

Characteristically GA_3 increased the main-stem length of young cotton plants in proportion to the total amount applied over the range of 10 to 1000 μg; 1.0 μg was ineffective. Dry weights of the stems and petioles, but not of the leaves, were consistently increased by the 10 and 100 μg doses. The 1000 μg dose, which had little or no effect on the dry weight of stems and petioles, drastically reduced leaf dry weight and caused pronounced chlorosis resembling nitrogen deficiency. It also reduced main-stem diameter and produced a condition resembling lodging.

Effects of GA_3 on the individual carbohydrate fractions were somewhat variable but total carbohydrates, as well as lignin, were each consistently increased in the stems and petioles as the level of GA_3 was increased; the leaves, except for increases at the inhibitory level, were essentially unaffected.

In the stems and petioles, but not in the leaves, total ash and total nitrogen contents (g/plant) were each increased by the growth promoting levels of GA_3 but suppressed in both tissue fractions by the inhibitory level (1000 μg).

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EFFECTS OF HIGH TEMPERATURE ON YIELD OF PEAS[1,2,3]

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High temperatures during the growing season have been suspected by growers and commercial canning companies to be associated in some way with reduced yield of *Pisum sativum* L. In some years in Minnesota high temperatures occur during May and June at which time plantings of the Alaska variety are at stages between flowering and maturity. Under these conditions yield may be reduced. Because of the influence of numerous other factors, the results obtained from a study of environmental factors in the field must be interpreted as the result of the interaction of a large number of uncontrolled factors. How much of the reduction in yield can be attributed to the effect of high temperatures alone and not to other factors such as mineral deficiencies (4, 7, 8), pathogenic organisms (5, 9, 11, 13), and genetic inheritance (1, 14) can be determined only by studying these effects under controlled conditions.

MATERIALS AND METHODS

For this study, plants were grown in a controlled light and temperature room. Light was provided by standard cool white fluorescent tubes and 60-watt incandescent bulbs. Light intensity averaged 1500 ft-c at plant top height.

The temperature of the room was maintained at 24° ± 1° C (ca 75° F) during a 12-hour light period followed by a 12-hour dark period at 18° ± 1° C (ca 65° F). The relative humidity, measured by a recording hygrothermograph, was 45 ± 3 % during the light period and 97 ± 3 % during the dark period.

The high temperature treatments were obtained by placing pea plants at selected stages of development and for different lengths of time in a cabinet comprised of three chambers maintained at 27° C (ca 80° F), 29° C (ca 85° F), or 32° C (ca 90° F). The cabinet was heated by resistance wire that lined the walls of each chamber, and to some extent, by the lights. The treatment temperatures varied ± 0.5° C at bloom node height of the peas in each of the three chambers. The temperature of the soil in the room and in the chamber was fairly constant at 22° C (ca 72° F).

Twenty-four seeds of *Pisum sativum* L. var. Alaska from the 1955 crop were sieved for uniformity, treated with a fungicide, and planted in redwood boxes. The planting medium used was composed of three parts Minnesota sandy-loam, two parts sand, one part peat, and autoclaved for four hours at 121° C. A commercial fertilizer consisting of 10 % nitrogen, 52 % phosphorus as P_2O_5 and 17 % potassium as K_2O was applied each week at the rate of 150 ml of a 2.3 % solution to each box. The plants were thinned to 16 plants per box two weeks after planting and to 8 plants per box at full bloom (considered as the day during which the standard petals reflexed fully toward the calyx).

It was found in preliminary experiments that flowering could be predicted accurately by the number of heat units accumulated. Heat units are calculated for peas by taking the average number of Fahrenheit degrees of heat above 40° F to which the plant is exposed in a 24-hour period. Taking 40° F as the base temperature for peas (10), 30 heat units were accumulated daily. Flowering of Alaska peas under these conditions occurred after 780 heat units had accumulated or after 26 days. The stage five days before full bloom was accurately estimated to be when 630 heat units had accumulated or 21 days after planting.

Twenty-one days after planting, three boxes were placed in each of the three chambers (27°, 29°, and 32° C). The daily duration of treatment was 6 hours, beginning three hours after the lights came on and ending three hours before the lights went off (fig 1). After the 1st 6 hours of treatment, one box was removed from each of the three chambers and placed in the 24° to 18° C (75° to 65° F) environment for the remainder of the experiment. Of the remaining two boxes in each chamber, one was treated for two more periods and one box for four more periods. This same procedure was carried out 26, 31, and 36 days after planting. By these methods the effects of three temperatures (27°, 29°, and 32° C), three durations (1, 3, and 5 daily periods of 6 hours

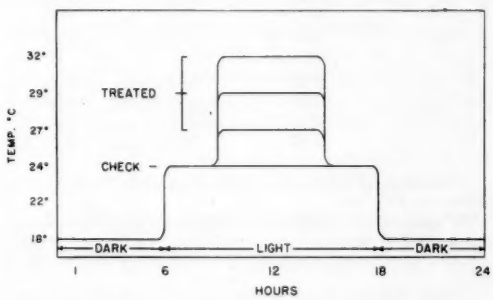

FIG. 1. Diurnal temperature cycle for both check and treated plants.

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[3] Contribution from the Department of Plant Pathology and Botany, Institute of Agriculture, University of Minnesota. Paper No. 3923, Scientific Journal Series, Minnesota Agricultural Experiment Station. Portion of a thesis presented by the senior author to the Graduate School of the University of Minnesota, in partial fulfillment of the requirements for the M.S. degree.

each), and four morphological stages of development (5 days prior to full bloom (−5), full bloom (FB), 5 days after full bloom (+5), and 10 days after full bloom (+10)) were studied. The plants were harvested 45 days after planting.

Three complete experiments were made in which the number of peas and the fresh weight of peas from each bloom-node were recorded. The data on fresh weight and number of peas from the 1st bloom-node were analyzed statistically by means of the "t'" test (12).

Experimental Results

Because of the large number of treatments and the similarity of results, only one of three time-replicated experiments will be discussed in detail.

The interaction of treatments at three temperatures for three durations of treatment during four stages of development can be shown by submerging one variable and by comparing graphs with a different variable submerged in each case.

The effect of the three main variables, temperature, duration of treatment, and stage of treatment on fresh weight of peas of canning size from the pod at the 1st bloom-node and from all bloom-nodes and on the number of peas of canning size from the pod at this node can be seen in table I. The fresh weight of peas from the 1st bloom-node was reduced in the order 32°, 29°, 27° C when duration of treatment and stage of the plants during heat treatment were submerged. When considering duration of treatment alone, five days of heat treatment was more detrimental than three days and three days more than one day. Those plants receiving their heat treatment beginning five days after full bloom (+5) were lower in fresh weight of peas from the 1st bloom-node than those plants treated during the other stages used in this experiment.

Plants beginning their treatment five days after full bloom at 27°, 29°, or 32° C and for one, three, or five days were lower yielding than comparable treatments at other stages (figs 2 A, 2 B, 2 D, and 2 E). Within each stage, fresh weight and number of peas from the 1st bloom-node was reduced more with increasing temperature from 27° to 29° to 32° C and with increasing duration from one to three to five days. In comparison with the fresh weight and number of peas from the pod at the 1st bloom-node of the check plants, those plants kept for one day at 27° C (80° F) were not affected, while the fresh weight and number of peas from plants treated for five days at 32° C (90° F) were substantially reduced (figs 2 C and 2 F). Within each temperature, the longer the treatment, the lower the yield and for each of the durations of treatment at high temperature, the higher the temperature, the lower the yield.

Whether the loss in fresh weight of peas from the pod at the 1st bloom-node was compensated for by a gain in weight of peas produced at higher nodes was determined by examining the total weight of peas produced at all nodes. The same general trends were found with fresh weight of peas from all bloom-nodes as were found with fresh weight of peas from the 1st bloom-node indicating that the peas produced at higher nodes did not compensate for the reduced yield at the 1st bloom-node (table I).

The present study has shown not only that temperatures of from 27° to 32° C (80° to 90° F) reduce fresh weight, and number of peas of canning size, but also has defined the effect of length of exposure to these temperatures and the stage of the plant most severely affected by these temperatures.

Table I

Average † Fresh Weight (FW) and Number of Peas from the First Bloom-node Pod and Average Fresh Weight of Peas from All Pods

Treatments	Mean FW peas from 1st node (grams)	Mean no. peas from 1st node	Mean FW peas from all nodes (grams)
Check	7.98	21.8	9.54
Temperature			
27° C (80° F)	7.34	20.3	8.88
29° C (85° F)	7.05	19.2	8.84
32° C (90° F)	6.43 **	18.2 *	8.22
Duration			
1 day	7.50	20.5	9.32
3 days	6.70 *	19.3	8.75
5 days	6.63 **	17.9 **	7.88 **
Stage ‡			
−5	7.20 *	20.0	9.12
FB	7.39	20.3	9.38
+5	6.15 **	16.8 **	7.45 **
+10	7.02	19.9	8.65

* Significantly lower than the checks by "t" test comparison at 5 % level.

** Significantly lower than the checks by "t" test comparison at 1 % level.

† Averages based on 5 to 12 boxes each containing 8 plants.

‡ Stage denotes those plants which received high temperature treatments beginning 5 days before full bloom (−5); at full bloom (FB); 5 days after full bloom (+5); or 10 days after full bloom (+10).

Discussion

In 1926, Boswell (2) summarized the results of three years of investigation on the influence of temperature on the growth and yield of garden peas. By planting peas at weekly intervals from the end of March to the middle of May, the peas were subjected to progressively higher temperatures during their development. At later plantings (higher temperatures) less time was required to reach certain stages of development and the weight of plant, weight and number of pods, and number of peas per plant was lower.

Boswell (3) later reported that the closest inverse relation between high temperature and yield was for the period from blossoming to harvest although from these investigations he could not point out any one critical period during which high temperatures were most detrimental. In one experiment in 1925 a

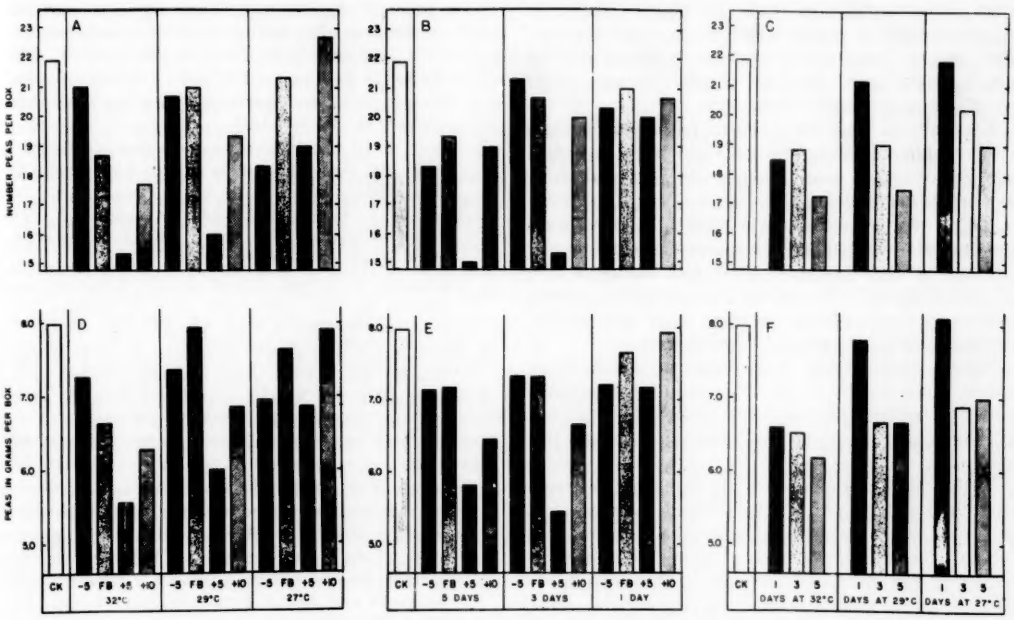

FIG. 2. Average number of peas (A, B, and C) and fresh weight of peas (D, E, and F) from 8 plants. Peas were harvested from the pod at the 1st bloom-node. Figs. 2 A and 2 D: Analysis of yield by temperature of treatment beginning at 4 morphological stages of development: 5 days before full bloom (−5); full bloom (FB); 5 days after full bloom (+5); and 10 days after full bloom (+10). Averages are based on 9 boxes (treated) or 5 boxes (check). Figs. 2 B and 2 E: Analysis of yield by duration of treatment beginning at the same 4 morphological stages. Averages based on 9 boxes (treated) or 5 boxes (check). Figs. 2 C and 2 F: Analysis of yield by temperature of treatment for 3 durations. Averages based on 12 boxes (treated) or 5 boxes (check).

delay in planting of 11 days after the first planting resulted in a decrease in yield of approximately 50 %.

Using controlled light and temperature conditions, Karr et al (6) studied the effect on Alaska peas of a 12-hour treatment at 32° C during the light period, of a 12-hour treatment at 24° C during the dark period, or a combination of both treatments. The yield of peas grown under these various conditions for three or four consecutive days was compared to the yield of peas grown at a temperature of 24° C during the light period and a temperature of 15.5° C during the dark period. The treatments were begun at various periods from 2 to 19 days after full bloom. They found that the greatest reduction in yield occurred in those plants receiving high temperature during both the light and dark periods. The critical period for those plants given high temperature treatments only during the light period was 9 to 11 days after full bloom; while the critical period for those given high temperature treatment only during the dark period was 6 to 9 days after full bloom. The critical period found by Karr et al (6) is similar to the period of from 5 to 10 days after full bloom found to be critical in the present study.

High temperatures may have reduced pea yield by causing an increase in respiration, decreasing the concentration of nutrients needed for ovule development, or by reducing translocation of materials into the pods and peas. High temperatures also may have interfered in some way with the balance of nitrogenous compounds and the synthesis of proteins. Other conditions such as the effect of high temperature on genetic expression, mineral nutrition, pathogenicity of organisms, or cell growth may have also influenced the yield of peas. Ovule failure can probably result from a number of causes. However, because of the complexity of conditions in a natural environment, it is often impossible to identify these causes. By means of a controlled environment, it has been possible to study the effect of temperature on yield of peas. The knowledge of the effects of a few short periods of high temperature on yield of peas will be useful to commercial companies in the breeding of heat-resistant varieties and in selecting better areas in which to grow peas.

SUMMARY

Treatment of Alaska peas with high temperatures during a period from five days before full bloom to 15 days after full bloom reduced fresh weight, and the number of peas which were classed as canning size.

All treatments at high temperatures were not equally effective in reducing yield of peas. When the data from plants receiving high temperature treat-

ments were examined by combining plants treated for different durations and at different stages of development, it was found that exposure to 32° C (90° F) reduced yield more than 29° C (85° F) and 29° C (85° F) more than 27° C (80° F).

Exposure to high temperature for 6-hour periods in the middle of the light period on five consecutive days reduced yield more than three days, and three days more than one day.

Plants treated at five days after full bloom were most severely affected by high temperature.

Plants treated five days after full bloom at 27°, 29°, or 32° C (80°, 85°, or 90° F) and for one, three, or five days were lower yielding than comparable treatments at other stages of development.

Within each of the four stages of development studied, fresh weight of peas was reduced more with increasing temperature from 27° to 29° to 32° C and with increasing duration from one to three to five days.

Within each temperature, the longer the treatment, the less the yield and for each of the durations, the higher the temperature, the lower the yield.

The practical application of results obtained in this study are discussed in view of breeding heat-resistant varieties of peas and selecting suitable areas for growing peas on a commercial scale.

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ORNITHINE, CITRULLINE, AND ARGININE METABOLISM IN WATERMELON SEEDLINGS[1]

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Arginine occurs widely both in the free form and in proteins of higher plants but there is little information on its metabolism in plants. The discovery and isolation of citrulline from the watermelon fruit by Wada (18), and the studies of Krebs and Henseleit (11) led to the formulation of the ornithine cycle. Since then much attention has been devoted to arginine metabolism in animals and a number of microorganisms (12), and its catabolic and anabolic reactions are in some cases well established. Coleman and Hegarty recently reported upon the metabolism of ornithine in potassium deficient barley (4, 5).

Ornithine, citrulline, arginine, and urea have been found in some plants but, except as cited above, their relationships to each other and metabolic interconversions have not been studied. The purpose of this investigation was to gain information concerning the interconversion of these compounds in the watermelon seedling.

[1] Received May 1, 1958.

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Methods

Watermelon seedlings (var. Klondike) were used for this study. Seeds previously treated with "Semesan" fungicide were soaked for 48 hours in aerated tap water and germinated on cheesecloth as described in an earlier publication (9). The combined sprouts and roots were vacuum infiltrated with solutions of labeled and unlabeled compounds. Extracts of the plant material were studied for both qualitative and quantitative differences.

Vacuum Infiltrations: Four to 10 g fresh weight of seedlings were cut into approximately 2-cm pieces

and immersed in solution in a vacuum desiccator. A vacuum of about 10-mm pressure was produced, held 10 to 15 minutes and air was allowed to re-enter the container rapidly. The procedure was repeated. The plant material was rinsed with water and placed in a moisture-saturated atmosphere in a north window for a suitable incubation period. The plant material was extracted with boiling 70 % ethanol and the extracts were prepared for analysis by evaporation to dryness and resolution in water as described previously (9). Non-labeled compounds, DL-ornithine monohydrochloride, L-citrulline, L-alanine, DL-aspartic acid, ammonium chloride, and guanidinoacetic acid were used as 0.01 M solutions in sufficient volume to immerse the plant parts. The labeled compounds prepared as 0.001 M solutions were: DL-ornithine monohydrochloride-2-C^{14}, L-arginine-C^{14} (Random), and urea-C^{14}.

HOMOGENATE PREPARATION: For experiments in which homogenates were used, material was prepared in a blender according to the procedure of Mueller, Quinn, and Reuckert (13). Twenty five to fifty grams of plant tissue were ground for two to three minutes in a chilled blender with an equivalent weight of water at 0° C. The resultant homogenate was filtered through several layers of cheesecloth and the filtrate used immediately.

ANALYTICAL METHODS: Qualitative comparisons were made with 1- and 2-dimensional chromatography using the methods and indicator reagents described (9). The chromatographic solvents most generally used were *N*-butanol : acetic acid : water (4 : 1 : 5), water saturated phenol, and 95 % ethanol : NH_4OH (95 : 5). Citrulline and arginine were separated from each other on chromatograms with the solvent system methanol : triethanolamine : water (80 : 4 : 16). The multiple descending chromatographic technique suggested by Block, et al, (2) with the non-aqueous phase from a mixture of *N*-butanol : phenol : acetic acid : water (20 : 20 : 8 : 40), was used to separate ornithine, lysine, histidine, glycine, serine, and aspartic acid.

In order to determine the specific activities of amino acids labeled with C^{14}, they were usually first separated by elution from a Dowex-50 (cation) column with hydrochloric acid according to the methods of Stein and Moore (14). The fractions that contained a particular amino acid were bulked and evaporated in vacuo at 50 to 60° C, or by hot air. The residues were further purified by band paper chromatography. The area containing the amino acid was cut out and eluted from the paper with water. Each amino acid was purified in this manner with at least two suitable solvents and the final products were characterized further on 2-dimensional chromatograms with color tests (9).

Arginine was recovered from infiltrated samples in some cases by precipitation from the extract solutions by the flavianic acid method of Vickery (16). Precipitations were carried out in small tapered centrifuge tubes and the precipitates were recovered by centrifugation. The washed precipitates were dissolved in 1 ml of 4 M HCl, further purified by band paper chromatography in butanol : acetic acid : water with subsequent recovery of arginine by elution from the paper with water. Citrulline was obtained from the supernatant solutions after the precipitation of arginine by adding excess $Ba(OH)_2$ to precipitate barium flavinate. Excess barium was removed as the carbonate and the solution was treated with 4 % phosphotungstic acid according to the procedure used by Wada (18). The supernatant solution was band chromatographed in each of the three solvents, phenol, butanol : acetic acid : water, and phenol : isopropanol : water (70 : 5 : 25). The product on chromatography in a number of solvents, gave single ninhydrin- and *p*-dimethylamino-benzaldehyde (7) spots and negative reactions to the vanillin (6) and Sakaguchi (1) tests.

The ninhydrin method of Troll and Cannon (15) was used for quantitative estimation of single amino acids for specific activity determinations. A standard curve and suitable blanks were run simultaneously for each amino acid. Duplicate determinations were made, which gave variations between duplicates of $\pm$ 10 %.

The quantities of ornithine and proline were measured by the method of Chinard (3). Citrulline was determined by the carbamidodiacetyl method of Knivett (10). Arginine was measured in plant extracts or purified solutions by a modification of the method of Sakaguchi (8).

RADIOACTIVITY MEASUREMENTS: Radioactivity determinations were made with a thin end-window Geiger tube. Aliquots of known concentration were evaporated on copper planchets and counted to give a probable counting error of 2 % or less. The weights of the compounds on the planchets were negligible so that no corrections were made for self absorption.

RESULTS

INFILTRATION OF UNLABELED COMPOUNDS: Preliminary qualitative and quantitative comparisons were made of extracts from seedlings infiltrated with citrulline, ornithine, or water. No appreciable effect of this treatment on the concentration of arginine was noted and the qualitative distribution of amino acids was unchanged except for the presence in the water infiltrated seedlings of an unknown Sakaguchi-positive substances having an R_f value on butanol chromatograms half that of arginine. This substance was absent in the citrulline and ornithine infiltrated samples.

INFILTRATION OF LABELED COMPOUNDS: Since preliminary studies with N^{15} labeled ammonium ion indicated that a comparatively rapid interconversion of these amino acids did take place, various treatments were made with C^{14} labeled ornithine, arginine, and urea.

DL-ORNITHINE-2-C^{14}: A number of amino acids were isolated from the ornithine-infiltrated seedlings and their specific activities determined. The results

TABLE I

SPECIFIC ACTIVITIES OF AMINO ACIDS ISOLATED FROM WATERMELON SEEDLINGS INFILTRATED WITH 0.001 M DL-ORNITHINE-2-C^{14} *

AMINO ACID	SPECIFIC ACTIVITY (CPM/μG-ATOM CARBON)	CONCENTRATION MICROMOLES/G FRESH WEIGHT
Ornithine	22,600	...
Citrulline	238	0.9
Arginine	36.6	0.9
Proline	36.0	0.2
Lysine	11.0	0.9
Glutamic acid	11.2	1.0
Alanine	10.3	2.6
Threonine	7.3	0.5
Glycine	10.5	0.9
Valine	1.6	0.7
Serine	< 5.0	1.5
Histidine	< 0.2	0.3
Aspartic acid	< 1.5	1.1

* Incubation time: 24 hr.

are tabulated in table I along with the concentrations of free amino acids found in the seedlings.

The majority of the amino acids contained less than 30 cpm per micromole radioactivity. Compounds known to be metabolically related to ornithine in other organisms were highly labeled. Citrulline contained the largest amount of radioactivity, about 6.5 times that of arginine on a per mole basis. In the seedlings there were approximately equal quantities of arginine and citrulline in the free form.

Glutamic acid and lysine were labeled to an intermediate degree. The specific activity of proline was about three times that of glutamic acid but the latter was present in concentrations five times those of proline.

L-ARGININE-C^{14} (U): Uniformly-labeled L-arginine was infiltrated and the specific activities of some of the amino acids, including ornithine and citrulline, were determined. After infiltration two lots were incubated 3 and 24 hours respectively. The results are presented in table II.

By the end of three hours appreciable activity had appeared in a number of amino acids. Ornithine was most highly labeled with a specific activity almost 50 times that of citrulline. Proline was also somewhat higher in activity than citrulline and lysine and histidine had significant labeling.

After 24 hours, considerable equilibration had taken place so that the differences between ornithine and citrulline were no longer evident.

Urea was isolated from the plant extract of the three-hour lot after the addition of 0.5 micromole of authentic urea. The compound was separated on a Dowex-50 column and further purified by band paper chromatography in butanol : acetic acid : water and *N*-butanol : 95 % ethanol : water (80 : 20 : 20) on Whatman no. 1 papers previously washed with 1 % oxalic acid and water. Paper chromatography with *p*-dimethylamino-benzaldehyde treatment of extracts from non-infiltrated seedlings showed there was less than 0.02 micromole urea per gram of seedlings. The urea isolated from the arginine-infiltrated seedlings contained a small but definite radioactivity, which was estimated to be 140 cpm per micromole of in situ urea.

Urea-C^{14} was infiltrated and after 24 hours incubation time the free amino acids were isolated and their specific activities determined. The results are shown in table III.

Appreciable quantities of the radioactive isotope appeared in a number of amino acids. The long incubation time required resulted in considerable randomization of carbon so that it is difficult to discern the pathway of entry. The comparatively high label in citrulline (approximately 8 times that of ornithine or arginine) certainly suggests that CO_2 fixation was primarily responsible for incorporation of the label.

EXPERIMENTS WITH HOMOGENATES: Additional experiments conducted with crude homogenates indicated that isolation of reactions involved in these interconversions is possible. The extent to which ornithine is converted to citrulline and arginine is far below the activity level of intact leaf disks. Transfer of label from ornithine to proline proceeds readily.

Efforts were made to evaluate the role of carbamyl phosphate in the synthesis of citrulline from ornithine by crude homogenates. The non-enzymatic formation of citrulline was sufficiently rapid, however, to mask any enzyme effects. A further evaluation of this and other reactions of the homogenates is contemplated.

DISCUSSION

Although ornithine and citrulline have been demonstrated to occur in a number of plants (9, 4, 5, 17) the metabolic role of these amino acids in the plant is not clear. Coleman and Hegarty (5) have demonstrated the conversion of carbamyl labeled citrulline to arginine by barley and white clover and it appears

TABLE II

SPECIFIC ACTIVITIES OF AMINO ACIDS ISOLATED FROM WATERMELON SEEDLINGS INFILTRATED WITH 0.001 M L-ARGININE-C^{14} (UNIFORMLY LABELED)

AMINO ACID	SPECIFIC ACTIVITY PER CARBON (CPM/μG-ATOM CARBON)	
	3 HR INCUBATION	24 HR INCUBATION
Arginine	12,500	1,150
Ornithine	547	163
Citrulline	11.2	150
Proline	52.0	...
Lysine	16.7	< 3.0
Histidine	14.3	< 3.0
Glutamic acid	2.5	16.6
Alanine	4.0	9.0
Threonine	...	18.0
Aspartic acid	...	6.0
Glycine	...	< 5.0
Serine	...	< 0.3

TABLE III

SPECIFIC ACTIVITIES OF AMINO ACIDS ISOLATED BY DOWEX-50 COLUMN AND PAPER CHROMATOGRAPHY FROM WATERMELON SEEDLINGS INFILTRATED WITH 0.001 M UREA-C^{14} *

AMINO ACID	SPECIFIC ACTIVITY (CPM/MICROMOLE)
Arginine	8.5
Ornithine	8.0
Citrulline	71
Alanine	320
Proline	320
Lysine	75
Aspartic acid	38
Serine	37
Glutamic acid	30
Leucines	28
Glycine	23
Histidine	22
Valine	39
Threonine	29

* Incubation time: 24 hr.

evident from the foregoing experiments that the complete sequence of Krebs-Henseleit conversion takes place in the watermelon seedling. Although both the formation and utilization of urea is demonstrated, it would appear unlikely that the role of this reaction sequence has much in common with its function in animals since the elimination of catabolic nitrogen via urea is not to be considered a normal plant function. It appears that ornithine and citrulline are intermediate in the synthesis of arginine in the plant, the latter presumably being utilized in protein synthesis. The role of arginase and urea formed by its action, however, remains obscure. Because with the specific activities a comparatively long incubation time was necessary to demonstrate significant conversion of infiltrated ornithine or arginine in the plant, a considerable randomization of the label occurred. Nevertheless, the distribution of activities observed after an appropriate incubation period serves to indicate that the reaction sequence is similar to that in animals. When isotopically labeled urea was infiltrated, the label appeared not only in citrulline to a considerable extent, but also in other amino acids which could be expected to become labeled as a result of CO_2 fixation. It is suggested that CO_2 from the breakdown of urea is responsible for the label appearing in arginine rather than a reversal of the arginase reaction. The disparity between the specific activities of proline and glutamic acid is not explained.

When seedlings were infiltrated with arginine and incubated for only three hours, proline was highly labeled in comparison with glutamic acid. The conclusion is implied that proline was derived from some intermediate other than glutamic acid, presumably glutamic semi-aldehyde, although this was not demonstrated directly. Moreover, it is impossible to ascertain from experiments of this type the comparative sizes of the various amino acid pools and the fractions of these pools which are metabolically active. Only the general close metabolic relationship between proline and citrulline can be inferred.

SUMMARY

1. Watermelon seedlings and homogenates were incubated with isotopically labeled ornithine, arginine, and urea in order to follow the course of synthesis and breakdown of Krebs-Henseleit amino acids in that plant.

2. A rapid interconversion of arginine, ornithine, and citrulline takes place. Urea is split from arginine and presumably is further hydrolyzed to CO_2 and ammonia. These can then be fixed with the formation of citrulline and arginine from ornithine via reactions analogous with those in animals.

3. On the basis of comparative specific activities, the close metabolic relationship of glutamic acid and proline to ornithine in higher plants is inferred. Specific activity of these amino acids precluded glutamic acid being intermediate between ornithine and proline.

4. Relationships between these reactions and similar reactions in animals and microorganisms are discussed.

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TRIPHOSPHOPYRIDINE NUCLEOTIDE DIAPHORASE FROM WHEAT GERM[1,2]

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The term "diaphorase" has been used in a variety of ways and consequently has taken on a number of different meanings. It was originally applied by Straub to a solubilized flavoprotein, obtained from mammalian heart muscle. The enzyme catalyzed the transfer of electrons from reduced diphosphopyridine nucleotide (DPNH) by way of flavin-adenine-dinucleotide (FAD) to an artificial electron acceptor, such as methylene blue or an indophenol derivative (6, 13, 19, 20, 21). The important point was that neither cytochrome c nor oxygen nor any other naturally occurring substrate could readily serve as an electron acceptor in place of the dyes. Subsequently, other purified flavoproteins have been found to oxidize pyridine nucleotides and to reduce dyes. In virtually all of these systems, however, natural substances could replace the synthetic acceptor. Thus the term "diaphorase activity" has been applied by some investigators to such enzymes as cytochrome c reductase when a dye was used as the electron acceptor in place of cytochrome. Strictly speaking, this use is not in keeping with the original designation of the term, since the enzyme can use a naturally occurring acceptor, namely cytochrome c.

The important problem concerning diaphorase, however, is the question of its role in the living cell. Minor modifications of Straub's isolation procedure have been found to yield a cytochrome c reductase (8, 16). Edelhoch et al (8) suggested that the native diaphorase protein may have cytochrome c reductase activity but that this is lost during the purification procedure of Straub. Mahler et al (16) have indicated that cytochrome c reductase and diaphorase proteins differ only in the steric arrangement around the site of binding cytochrome c to the enzyme. The differences were also tentatively ascribed to a removal of iron from the cytochrome c reductase to yield a diaphorase (14). While the relationship between these two systems is still not clear, it is possible that one is derived from the other.

Until very recently evidence for the occurrence of a reduced triphosphopyridine nucleotide (TPNH) diaphorase was almost completely lacking, Curran et al (6) having mentioned a very low activity of Straub's diaphorase with TPNH. Abraham and Adler (1) compared the DPNH- and TPNH-diaphorase activities of the enzyme prepared from heart muscle and adrenal gland according to Straub's purification procedure and observed very little activity of the enzyme with TPNH. Mahler et al (16) reported that TPNH-cytochrome c reductase will also reduce dyes. The first truly specific TPNH-diaphorase was found in spinach leaf chloroplasts by Avron and Jagendorf (2, 3). Evans (9) reported on a TPNH-diaphorase from soybean leaves at the 1956 American Institute of Biological Sciences meetings.

The present paper elaborates on an earlier report (4) given at the same meetings describing the purification and characterization of a TPNH-specific diaphorase from wheat germ. A DPNH-specific diaphorase is also present. There appears to be no requirement for flavin or metallic ions. Evidence is also presented to show that a heat stable factor(s) in wheat germ will convert the TPNH-diaphorase and the DPNH-diaphorase into enzymes having cytochrome c reductase activity. Menadione (the nucleus of vitamin K) and a number of other quinones can also serve in place of the naturally occurring substance(s).

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[2] Contribution No. 235 of the McCollum-Pratt Institute. This investigation was supported in part by a research grant (RG-2332) from The National Institutes of Health, U. S. Public Health Service.

Materials and Methods

Wheat germ was generously provided by General Mills Inc. DPN and TPN of 90 % purity were obtained from the Pabst Laboratories. They were reduced enzymatically by the method of Pullman et al (18) and Nason and Evans (17), respectively; or non-enzymatically by treatment with sodium hydrosulfite (10). Horse heart cytochrome c of approximately 60 % purity, FAD of approximately 40 % purity, and flavin-mononucleotide (FMN) were obtained from the Sigma Chemical Company. The menadione and other quinones were purchased from the Eastman Kodak Company unless otherwise indicated.

Diaphorase Assay: The reaction mixture was routinely composed of the following: 0.05 ml of enzyme (usually diluted about 1 : 10); 0.85 ml of 0.1 M tris-(hydroxymethyl)-amino-methane (TRIS) buffer, pH 8.0; and 0.05 ml of 3×10^{-4} M 2,6-dichlorophenol-indophenol. At zero time 0.05 ml of TPNH or DPNH (1.15 micromoles per ml) was added and the decrease in optical density at 645 mμ was measured at 30-second intervals for five minutes. A unit of enzyme activity is defined as that quantity which will cause a decrease in Log I_o/I of 0.001 in the interval from one to three minutes. The rate of indophenol dye reduction is directly proportional to the protein concentration under these conditions of measurement (fig 1). Specific activity is defined as units of enzymatic activity per mg of protein. Protein was determined by the procedure of Lowry et al (12) standardized with crystalline bovine serum albumin.

Assay for Cytochrome c Redution: The reaction mixture was routinely composed of the following: 0.05 to 0.1 ml of the enzyme; 0.1 ml of 0.01 M KCN; 0.05 ml of 2 % aqueous cytochrome c; and enough 0.1 M TRIS buffer, pH 7.9, to give a volume of 0.95 ml. At zero time, 0.05 ml of TPNH or DPNH (1.15 micromoles per ml) was added and the increase in optical density at 550 mμ was measured at 30-second intervals for five minutes.

Assay for Reduced Pyridine Nucleotide Oxidase: The reaction mixture was routinely composed of the following: 0.05 to 0.1 ml of enzyme, and enough TRIS buffer, pH 7.9, to give a volume of 0.95 ml. At zero time, 0.05 ml of TPNH or DPNH (1.15 micromoles per ml) was added and the decrease in optical density at 340 mμ was measured at 30-second intervals.

Results

Purification of TPNH Diaphorase: All the steps in the purification, summarized in table I, were carried out at 0 to 4° C. Wheat germ (usually 10 to 20 g) was homogenized with 10 times its weight of 0.1 M phosphate buffer, pH 7.5, in a Ten Broeck tissue grinder. The homogenate (Fraction 1) was centrifuged at 3000 × G for 30 minutes, and the resulting supernatant solution (Fraction 2), designated as the crude extract, in turn centrifuged at 140,000 × G in

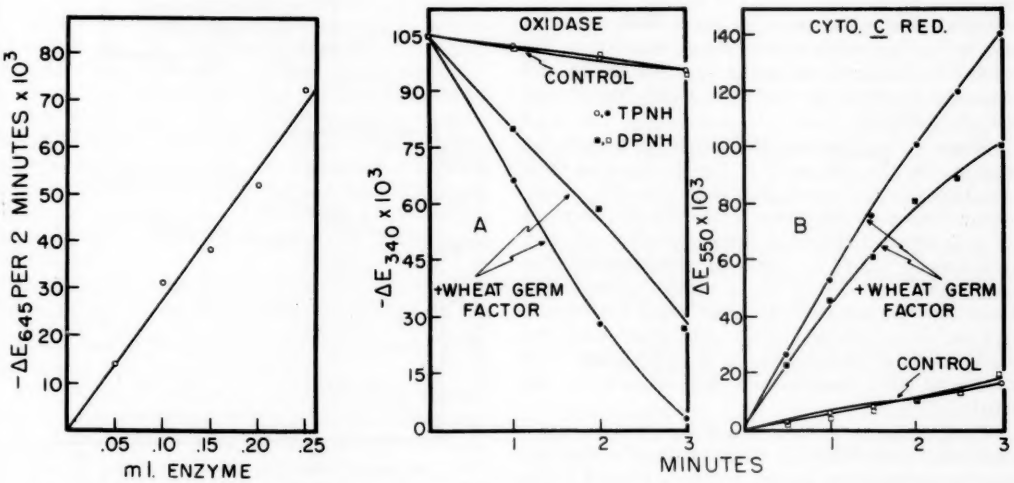


Fig. 1 (*left*). Effect of increasing enzyme concentration on rate of reduction of dye (diaphorase activity). The enzyme preparation contained 1.14 mg protein per ml.

Fig. 2 (*center and right*). Effect of added "wheat germ factor" on pyridine nucleotide oxidase and cytochrome c reductase activities. (A) TPNH- and DPNH-oxidase activity. Cuvette contained 0.5 ml 0.1 M TRIS buffer, pH 8.0, 0.4 ml "wheat germ factor," 0.05 ml enzyme (19.3 mg protein per ml) and 0.05 ml TPNH or DPNH (1.15 micromoles per ml) added at zero time. Control contained the same except 0.9 ml buffer and no factor. (B) Cytochrome c reductase activity. Cuvette contained 0.35 ml 0.1 M TRIS buffer, pH 8.0, 0.05 2 % cytochrome c, 0.1 ml 0.01 M KCN, 0.4 ml "wheat germ factor," 0.05 ml enzyme (19.3 mg protein per ml) and 0.05 ml TPNH or DPNH (1.15 micromoles per ml) added at zero time. Control contained the same except 0.75 ml buffer and no factor.

TABLE I

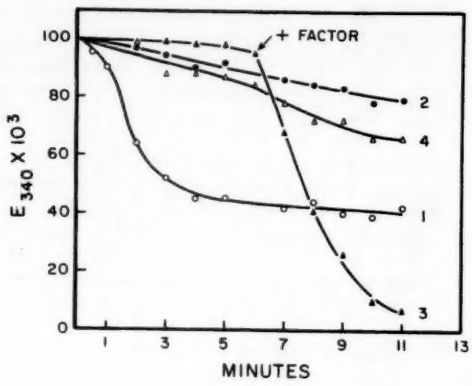
SUMMARY OF PURIFICATION OF WHEAT GERM TPNH DIAPHORASE

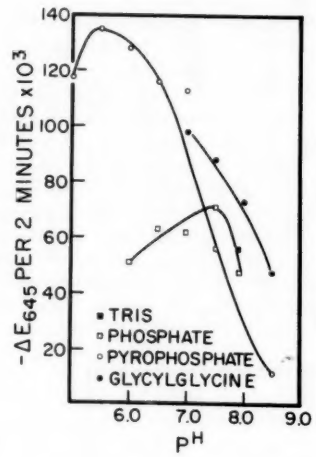
FRACTION	TOTAL UNITS $-\Delta E_{645}$ PER 2'	TOTAL PROTEIN MG	SPECIFIC ACTIVITY, UNITS PER MG PROTEIN	PERCENT RECOVERY
1. Crude homogenate	3,454	4400	785	..
2. Crude extract	3,352	3528	950	97
3. High speed supernatant	1,411	2250	627	41
4. Dialyzed supernatant	1,453	2516	975	42
5. Calcium phosphate gel supernatant	627	94	6666	18

the Spinco preparative centrifuge for one hour. The subsequent high speed supernatant solution (Fraction 3) was dialyzed against 100 to 150 volumes of 0.001 M TRIS buffer, pH 7.9, for 18 to 48 hours and the dialysate (Fraction 4) added to an equal volume of calcium phosphate gel (11) aged 9 months or longer (21 mg per ml). After 10 to 15 minutes of intermittent stirring, the mixture was centrifuged at 3000 ×G for 10 minutes and the supernatant solution (Fraction 5) collected as the final fraction. Further purification by means of ammonium sulfate fractionation failed to yield preparations of higher specific activity. As shown in table I, Fraction 5 represents a 10-fold purification over the crude extract with a recovery of approximately 20 % of the activity. A number of similar preparations was obtained by this procedure in which the degree of purification varied from 5- to 15-fold.

STABILITY OF ENZYME: Fraction 3 can be dialyzed or stored in the cold at 4° C for 48 hours or more with virtually no loss of diaphorase activity. Some preparations after treatment with calcium phosphate gel and ammonium sulfate were also dialyzed with little loss of activity. Dialyzed preparations have been stored at −15° C for a month without loss. These older preparations, however, when thawed and kept in an ice bucket in the laboratory may lose nearly half their activity during 8 to 10 hours. Ten minutes at 60° C resulted in a 50 % reduction of the activity.

RELATIONSHIP OF DIAPHORASE TO CYTOCHROME c REDUCTASE AND "WHEAT GERM FACTOR": The enzyme preparations designated Fractions 1 and 2 (table I), and sometimes also Fraction 3, not only had diaphorase activity, but also showed significant TPNH- and DPNH-oxidase and cytochrome c reductase activities. With further purification, however, the latter activities at times decreased, but could always be strikingly increased by the addition of boiled crude extract to the reaction mixture. Partial purification of the factor(s) responsible for this enhancing effect has been achieved by slurrying the supernatant solu-





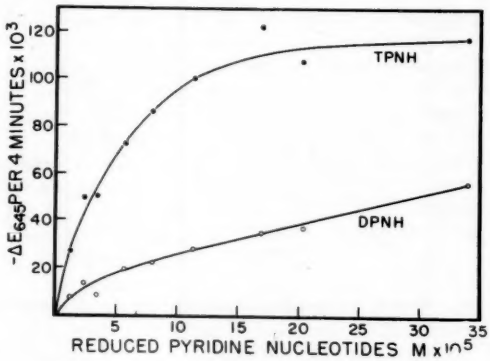


FIG. 3 (*top*). Effect of adding "wheat germ factor" after the start of the TPNH-oxidase reaction. Cuvette 1 (curve 1) contained 0.5 ml 0.1 M pyrophosphate buffer, pH 7.0, 0.4 ml "wheat germ factor," 0.05 ml enzyme (23 mg protein per ml) and 0.05 ml TPNH (1.15 micromoles per ml) added at zero time. Other cuvettes (curves 2, 3, 4) were similar but contained 0.9 ml buffer and no factor. At 6 minutes 0.4 ml of factor and 0.4 ml 0.1 M

tion of a 10-minute-boiled crude extract (Fraction 2) with animal charcoal in proportions of 10 ml per gram for approximately 10 minutes and filtering. The filtrate contained the active substance(s) which has been tentatively designated as the "wheat germ factor." Although as yet unidentified, it is an organic compound(s) since the ashed extract is without effect. Figure 2 shows an increased oxidase activity with added "wheat germ factor" of about 7- and 10-fold over the controls using DPNH and TPNH respectively, and increased cytochrome c reductase activities of 5- and 7-fold. Without the added factor, there is virtually no cytochrome c reductase activity. In effect the addition of the "wheat germ factor" appears to have transformed true diaphorase to cytochrome c reductase. There was little or no non-enzymatic reduction of cytochrome c by the factor. Figure 3 demonstrates the effect on TPNH oxidase activity of adding the "wheat germ factor" after the start of the reaction.

TPNH DIAPHORASE AND DPNH DIAPHORASE AS SEPARATE ENTITIES: That TPNH diaphorase and DPNH diaphorase are different enzymes was indicated by the fact that the ratio of their activities varies widely in different fractions as shown in table II. The ratio of TPNH diaphorase to DPNH diaphorase ranged from 0.5 to nearly 5, a range of 10-fold.

pH OPTIMUM: The variation of TPNH diaphorase activity with pH and buffer is represented in figure 4. Pyrophosphate proved to be the most effective buffer with a pH optimum at about 5.5. Phosphate buffer was about half as effective and gave optimal activity at pH 7.5. TRIS was more satisfactory than phosphate. In the more acid range there is some non-enzymatic reduction of the dye. With pyrophosphate at pH 5.5 it amounted to only an optical density change of 0.020. The curve in figure 4 has been corrected for the non-enzymatic reaction. Unfortunately, pyrophosphate buffer had not been tried until late in the investigation, so that most of the experiments had already been performed with TRIS buffer as indicated.

PYRIDINE NUCLEOTIDE AFFINITY: The data in figure 5 indicate a marked affinity for TPNH as the electron donor in the diaphorase system. The dissociation constant (K_m) of the enzyme-TPNH complex as estimated from the curve is about 5×10^{-5} M. The activity with DPNH is probably due to the presence of the DPNH diaphorase, since some enzyme preparations have been obtained which used only TPNH.

EFFECT OF FLAVINS: Thus far it has not been possible to demonstrate a requirement for flavin by the TPNH- and DPNH-diaphorase using added FAD or FMN in final concentrations ranging up to 10^{-4} M. Nor was there an effect of added flavin on the TPNH- and DPNH-oxidases in any of the fractions tried. Table III demonstrates the lack of relationship of wheat germ TPNH-diaphorase activity to the FAD content of the enzyme. As can be seen, the enzyme with the highest specific activity had no detectable FAD.

EFFECT OF METAL IONS AND METAL BINDING AGENTS: With respect to other possible activators of

TABLE II

RELATIVE ACTIVITIES OF WHEAT GERM TPNH- AND DPNH-DIAPHORASE IN VARIOUS PROTEIN FRACTIONS

TREATMENT	$-\Delta E_{645}$ PER ALIQUOT PER $2' \times 10^3$		RATIO OF ACTIVITIES TPNH/DPNH
	TPNH-DIAPHORASE	DPNH-DIAPHORASE	
Crude homogenate	137	63	2.17
Crude extract	152	83	1.83
High speed supernatant	130	54	2.41
Dialyzed supernatant	124	40	3.1
Calcium phosphate gel supernatant	6	11	0.55
Calcium phosphate gel precipitate	13	24	0.54
High speed supernatant undialyzed, calcium phosphate gel supernatant	65	14	4.36

TABLE III

LACK OF RELATIONSHIP BETWEEN WHEAT GERM TPNH-DIAPHORASE ACTIVITY AND FAD CONTENT OF THE ENZYME

ENZYME FRACTION	SPECIFIC ACTIVITY *	MICROMOLES FAD/MG PROTEIN **
1	944	1.6×10^{-5}
2	534	2.2×10^{-5}
3	6200	0
4	81	1.2×10^{-5}
5	0	1.7×10^{-5}

* $-\Delta E_{645} \times 10^3$ per mg protein.

** We are indebted to Dr. Chester DeLuca who measured the FDA content of the various fractions by an assay system involving the use of the apoenzyme of pig kidney D-amino acid oxidase coupled to lactic dehydrogenase (7).

phosphate buffer, pH 7.5, were added to cuvettes 3 and 4 respectively (curves 3 and 4).

FIG. 4 (*center*). Effect of pH on TPNH-diaphorase activity. Cuvette contained 0.85 ml of the buffer indicated (0.1 M), 0.05 ml 3×10^{-4} M 2,6-dichlorophenol-indophenol and 0.05 ml enzyme (5.7 mg protein per ml). At zero time 0.05 ml TPNH (1.15 micromoles per ml) was added.

FIG. 5 (*bottom*). Nucleotide saturation curves in diaphorase activity. The cuvettes contained 0.05 ml enzyme (11 mg protein per ml), 0.05 ml 3×10^{-4} M 2,6-dichlorophenol-indophenol and enough 0.1 M TRIS buffer, pH 8.0, to make a final total volume of 1.0 ml. At zero time varying quantities of TPNH or DPNH (1.15 micromoles per ml) were added to make the final concentrations indicated on the abscissa.

the diaphorase, it was not possible to show any enhancing effect of added metal ions, including Fe^{++}, Fe^{+++}, Zn^{++}, Mn^{++}, Mg^{++} or Cu^{++} at 10^{-5} M final concentration. Added cytochrome c had no effect on the TPNH- or DPNH-oxidase activities. Nor could measurable cytochrome c oxidase be detected in our preparations. That these oxidases do not involve a cytochrome oxidase pathway was further indicated by the observation that cyanide and azide at 10^{-3} M final concentration had no significant inhibitory effect. These compounds as well as salicylaldoxime, thiourea, versene or dimethylglyoxime at final concentrations of 10^{-3} M did not inhibit the diaphorase.

Effect of Various Compounds in Transforming Diaphorase Activity to Cytochrome c Reductase Activity: Table IV shows the effect of various compounds in transforming TPNH-diaphorase activity to cytochrome c reductase activity. The most effective was *p*-xyloquinone, followed by menadione, then the indophenol dye and *p*-benzoquinone. Apparently a methyl group is necessary for the activity of 1,4-naphthoquinones, since the latter is ineffective whereas the methyl derivative (menadione) functions well. It is also of interest that the presence of chlorine atoms on the benzoquinone ring results in a complete loss in activity. Tocopherol (not shown) was ineffective.

The action of menadione in restoring cytochrome c reductase and reduced pyridine nucleotide oxidases was further examined. Figure 6 shows that with the given amounts used, menadione increased TPNH-oxidase 15-fold over the control, as compared to a 5-fold increase by the charcoal treated "wheat germ factor." In the DPNH system, however, the effects were about the same with both substances, namely a 2- to 3-fold increase. It should be emphasized that these substances had little or no effect when the enzyme was omitted from the reaction mixture. Figure 7 demonstrates the effects of increasing concentrations of menadione on cytochrome c reductase and TPNH-oxidase. The dissociation constants (K_m) for

Table IV

Effect of Various Compounds in Transforming TPNH-Diaphorase Activity to Cytochrome c Reductase Activity

Compound	Concentration	$E_{550} \times 10^3$ per 2′
Control	.....	4
Menadione (2-methyl-1,4-naphthoquinone)	5×10^{-5} M	117
1,4-Naphthoquinone	3×10^{-4}	0*
1,2-Naphthoquinone	3×10^{-4}	5
1,2-Naphthoquinone sulfonic acid	3×10^{-4}	0
p-Benzoquinone	3×10^{-4}	52*
p-Xyloquinone	3×10^{-4}	148
2,5-Dichloroquinone	3×10^{-4}	0*
2,6-Dichloroquinone	3×10^{-4}	20*
Cystine	3×10^{-4}	6
Oxidized glutathione	3×10^{-4}	14
FAD	5×10^{-7}	7
FMN	2×10^{-5}	3
2,6-Dichlorophenol-indophenol	2×10^{-6}	75*
Methylene blue	0.005 %	31*

* Corrected for non-enzymatic rate.

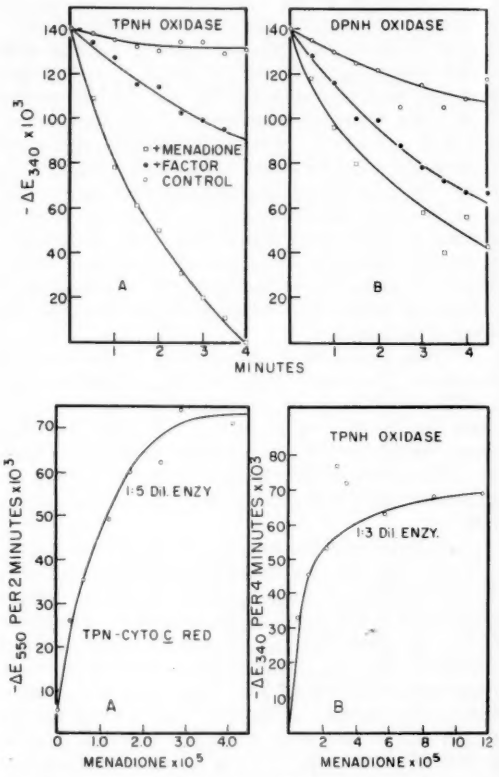


Fig. 6 (*top*). Effect of added "wheat germ factor" and menadione on TPNH- and DPNH-oxidase activity. Cuvette contained 0.4 ml "wheat germ factor" or 0.4 ml menadione (5 mg per 50 ml), but the controls contained neither of these, 0.1 ml enzyme (0.34 mg protein per ml) and enough 0.1 M TRIS buffer, pH 7.9, to make 0.95 ml. At zero time 0.05 ml TPNH or DPNH (1.15 micromoles per ml) was added.

Fig. 7 (*bottom*). Menadione saturation curves. (A) TPNH-cytochrome c reductase activity. Cuvette contained 0.05 ml enzyme (1.1 mg protein per ml), 0.1 ml 0.01 M KCN, 0.05 ml 2 % cytochrome c, varying quantities of menadione (1 mg per 50 ml) to make the final concentrations indicated on the abscissa and enough 0.1 M TRIS buffer, pH 7.9, to make a total volume of 0.95 ml. At zero time 0.05 ml TPNH (1.15 micromoles per ml) was added. (B) TPNH-oxidase activity. Cuvette contained 0.05 ml enzyme (1.9 mg protein per ml), varying amounts of menadione (5 mg per 50 ml) to make the final concentrations shown on the abscissa and enough 0.1 M TRIS buffer, pH 7.9, to make a total volume of 0.95 ml. At zero time 0.05 ml TPNH (1.15 micromoles per ml) was added.

the enzyme-menadione complexes as approximated from the saturation curves are the same, namely about 1×10^{-5} M.

Discussion

There are a number of possibilities with regard to a role for diaphorase in the living cell: (a) the diaphorase, during the course of its isolation and purification, has been modified or denatured in such a manner that it is no longer capable of catalyzing the reduction of its natural acceptor, perhaps due to the loss of a necessary cofactor or destruction of an active center on the enzyme; (b) the natural electron acceptor has not yet been found, or if already discovered, not yet tested in this system; or (c) diaphorase is an artificial model system, possibly inadvertently formed during mechanical disruption of tissues, with no bearing whatsoever on the metabolism of the living cell.

The present work indicates that cell-free preparations of wheat germ contain a naturally occurring substance which is responsible for transforming diaphorase activity to cytochrome c reductase activity. A TPNH-specific diaphorase and DPNH-specific diaphorase are involved. The fact that certain aryl compounds including quinones have a similar effect suggests that quinone-type compounds in wheat germ may be the responsible agents. The possibility exists, however, that quinone-type compounds are not present at effective levels in living cells but are formed in cell homogenates as a result of oxidative phenomena, subsequently acting as an artificial carrier much like an added dye. The current experiments, therefore, would not appear to favor any of the above possibilities for a function of diaphorase.

The mode of action of the quinones in converting diaphorase activity to cytochrome c reductase activity involves the enzymatic transfer of electrons from reduced pyridine nucleotides to the quinone (23, 24), followed probably by a non-enzymatic reduction of cytochrome c by the hydroquinone (15). As an alternative possibility, the latter step may be enzymatic in view of the recent demonstration by Colpa-Boonstra and Slater (5) of a reduced menadione oxidase in heart muscle preparations. Mahler et al (15) were able to convert Straub's diaphorase to a cytochrome c reductase by including menadione or certain other quinones in the reaction mixture. Weber and Brodie (22) have also recently indicated the presence of a "menadione-dependent cytochrome c reductase in *Mycobacterium phlei*."

In the absence of cytochrome c, the enhanced TPNH and DPNH oxidase activities with added "wheat germ factor" or quinone (e.g., menadione) is very likely due to an increased enzymatic rate of electron transport from the reduced pyridine nucleotide to the quinone followed by a rapid auto-oxidation of the latter as indicated in the following sequence:

$$\text{TPNH} + \text{H}^+ + \text{quinone} \xrightarrow{\text{enzyme}} \text{TPN}^+ + \text{hydroquinone}$$

$$\text{hydroquinone} + \text{O}_2 \longrightarrow \text{quinone} + \text{H}_2\text{O}_2$$

The wheat germ diaphorase can also be regarded as a menadione reductase (24) in view of its property of catalyzing the reduction of vitamin K_3. The apparent lack of a flavin requirement could be due to the presence of a tightly bound flavin nucleotide.

Summary

A TPNH-specific diaphorase has been purified approximately 10-fold from wheat germ. The enzyme is associated with a DPNH-specific diaphorase, but the relative activities of the two enzymes vary so widely in different protein fractions that they are considered to be separate systems. There appears to be no requirement for added flavin or metal ions. Metal binding agents have no effect on enzyme activity. The addition of a heat-stable organic factor obtained from a crude extract of wheat germ results in a 7- to 10-fold increase in cytochrome c reductase and oxidase activities of the enzyme. Several quinone-like compounds also served to transform the diaphorase to a cytochrome c reductase as well as to a TPNH-oxidase. The possible significance of diaphorase in biological systems is discussed.

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ACTION SPECTRUM OF PHOTOTROPIC TIP-CURVATURE OF AVENA[1,2,3]

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Previous determinations of action spectra of phototropism in plants have led to the following generalizations: (a) the photoreceptor consists of one or more yellow pigments, since the response is limited to wavelengths shorter than 500 mμ, and (b) β-carotene and riboflavin are considered the most probable candidates for the photoreceptor, since they are universally present in plant tissues and their absorption spectra coincide reasonably well with the action spectra. The absorption spectrum of β-carotene in organic solvents shows a principal peak at 445, a smaller one at 475, and a shoulder at 420 mμ. These closely match the action spectrum obtained by Johnston (8) for Avena in which he found a principal peak at 440 mμ and a second one at 475 mμ. Galston and Baker (7) obtained peaks at 450 and 470 mμ and Bünning (3) at 445 and 480 mμ, with a slight shoulder about 430 mμ. Riboflavin has a single, broad absorption peak in the region of 445 mμ and a near-ultraviolet peak at 370 mμ. Bünning (2, 3, 4) has proposed a dual pigment hypothesis in which β-carotene is a passive absorber which attenuates the radiant energy in its passage to the distal side of the coleoptile, while some other photochemically active pigment such as riboflavin is the actual photoreceptor.

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In rapidly growing shoots, auxin is a limiting factor for cell elongation and, therefore, various hypotheses have been presented based on the photooxidation of indoleacetic acid or active auxin sites. In vitro, β-carotene is not a strong photosensitizing agent. On the other hand, riboflavin is a very strong photosensitizer. These considerations led Galston and Baker (7) to study the photoinactivation of indoleacetic acid in pea-epicotyl breis. The spectral sensitivity of the inactivation had a broad peak at 440 mμ, with a sharp rise beginning in the near-ultraviolet. Because of instrumental difficulties, they were unable to extend the observations below 400 mμ. On the basis of these and other results, they proposed the possibility that a flavoprotein might be the photoreceptor system.

The principal criticism of any conclusions which are based upon the use of cellular macerates is that the structural organization of the cell is destroyed and pigments and substrates which might otherwise not be associated with one another may be brought together in close proximity and carry out completely abnormal reactions. This variable behavior of a photosensitizing pigment has been amply demonstrated in the case of the so-called "photodynamic action" of chlorophyll. Chlorophyll is well known for its role as the photoreceptor pigment in carrying out photosynthesis in the intact chloroplast. However, chlorophyll from tissue breis, in the presence of oxidizable substrates and oxygen, behaves as any other fluorescent pigment in accelerating substrate oxidation. The classic example is the hemolysis of the red blood cell (1).

The present study is an attempt to contribute to a resolution of the question of carotenoid versus flavin participation in phototropism by extending the action spectrum into the near-ultraviolet. Because of the relatively great difficulty of obtaining sufficient intensity with high spectral purity in the near-ultraviolet, none of the action spectra, except for those reported by Curry and Thimann (6), have been extended into the near-ultraviolet with any reasonable degree of resolution. Although both riboflavin and β-carotene have somewhat similar absorption bands in the blue, that for β-carotene in organic solvents continues to fall to a minimum in the near-ultraviolet, whereas that of riboflavin in aqueous solutions rises to a maximum at about 370 mμ. Also, β-carotene has three bands in the visible while riboflavin has only one. These characteristics should make it possible to discriminate between β-carotene and riboflavin if either one or the other is acting solely as the active pigment in the phototropic system.

Methods and Materials

Irradiation Equipment: The general plan of the irradiation equipment is given schematically in figure 1. The equipment was housed in a system of four cabinets containing: (a) the source and filters, (b) the grating monochromator, (c) holders for the Avena seedlings, and (d) source and holders for shadowgraphing. Various sources were tried in preliminary experiments, including both incandescent projection lamps and a 1 KW xenon arc. A modified automatic-feed, 1 KW cored-carbon projection arc produced from two to three times the intensity of the xenon arc in the near-ultraviolet. By careful adjustment, it was possible to obtain very satisfactory stability over the irradiation times that were used. All of the final work reported here was carried out with the carbon arc. The arc was of the reflector, axial-trim type operating at 60 volts, 15 amperes DC. The mirror was removed and the arc turned around and mounted at about 45 degrees to the optical axis so that the image of the crater of the positive carbon could be focused with a plano-convex quartz lens on the monochromator slit. The quartz lens was 7.5 cm in diameter and had a focal length of 10 cm.

Wavelengths longer than 625 mμ were removed by an aqueous solution filter (F_1) of hydrated copper sulfate (200 g/l, 5 cm pathlength). A second solution filter (F_2) of 3 cm pathlength usually was used to further reduce scattered energy. This filter consisted of various dye solutions (11) as given in table I. The composition was varied to accommodate the wavelength range in use.

The monochromator was designed and constructed in the laboratory shop. It was of relatively simple construction, consisting of a Littrow mounting with a double slit. The energy from the source entered the upper slit and the rays diverged to the parabolic off-axis mirror, M, which directed a parallel beam to the grating, G. The dispersed rays were then reflected back to the parabolic mirror and brought to a focus on the lower slit. The monochromatic energy was then reflected by the mirror, M′, into the irradiation chamber. The grating was rotated by a sine linkage driven by a precision screw, coupled to a revolution counter. The length of the sine linkage arm was so adjusted that the revolution counter read directly in millimicrons. Calibration with a mercury arc spectrum demonstrated that the system could track with an accuracy of better than 1.0 mμ throughout the visible and near-ultraviolet. The dispersing element was a Bausch and Lomb 100 × 100 mm, 600 lines per mm replica, plane grating, blazed for the near-ultraviolet. The linear dispersion ($dl/d\lambda$) was 3.0×10^5 (0.30 mm/mμ). The intensity of the emergent beam was controlled by adjusting the width of the double slit system and by varying the filter densities. The widths of the entrance and exit slits were varied from 1.0 to 4.0 mm, producing band widths of from 3 to 12 mμ for the minimum and maximum intensities used.

A standard blue source (S_1) was used to induce curvatures in a set of control seedlings to insure that the physiological behavior was the same for each

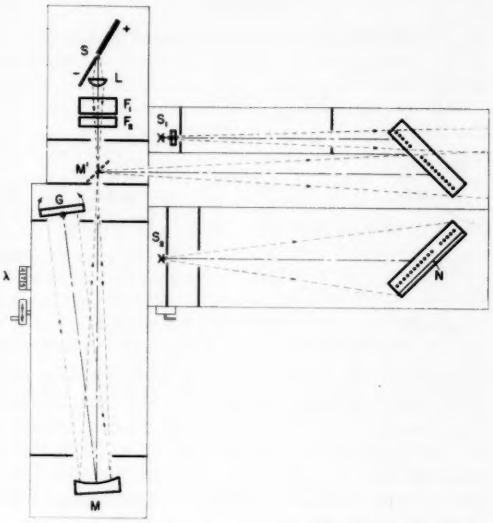

Fig. 1. Schematic diagram of monochromator and irradiation cabinets.

F_1 —Aqueous $CuSO_4 \cdot 5\,H_2O$ filter (5 cm pathlength)
F_2 —Aqueous dye filter (3 cm pathlength)
G —Plane grating (100 × 100 mm)
L —Quartz condensing lens (6 cm diameter, 10 cm focal length)
λ —Revolution counter (read directly in mμ)
M —Parabolic mirror (15 cm diameter, 1 m focal length)
M′—Plane mirror (20 × 20 mm)
N —Holder for seedlings and photographic film (5 × 6 × 25 cm)
S —Carbon arc
S_1 —Standard blue source
S_2 —Green source for shadowgraphing

TABLE I

COMPOSITION OF AQUEOUS DYE FILTERS

WAVELENGTH STATIONS Mμ	CONCENTRATION MG/L	DYE
350–390	50	New Fuchsin
400–450	170	Victoria pure blue BO
	30	Crystal violet extra pure APNX
460–480	60	Victoria pure blue BO
	500	8-hydroxy-quinoline sulfate
490–520	170	Picric acid

experiment. The source was a 75-watt, 120-volt projection lamp operating at 90 volts AC from an electronic voltage regulator. The beam passed through a diffusing glass plate, a 1.0-cm cell of 10 % hydrated copper sulfate and a crystal-violet gelatin filter (11). This system isolated a band of wavelengths from about 400 mμ to 500 mμ.

A green source (S_2) was used for shadowgraphing. This consisted of a small incandescent lamp with an image of the filament focused on a shutter with a condensing lens. A type B green safelight gelatin filter (12) transmitting from 520 to 580 mμ was placed in front of the aperture. This system produced an intensity of relatively inactive green energy at the film strip of 70 μw/cm². An exposure of 10 seconds produced distinct images with Kodak ortho type II film. It was found that a 10-minute exposure to the green safelight did not produce any measurable curvatures in the Avena seedlings.

Irradiances above 1 μw/cm² were measured with a vacuum thermopile which was calibrated frequently against a standard lamp from the National Bureau of Standards. The thermopile had a calcium fluoride window and operated directly into a Liston-Becker type modulation amplifier. A strip chart recorder was used to record the deflections. While it was not essential that a recorder be used, it greatly facilitated the evaluation of thermal drift in the thermopile system during measurement. Irradiances below the range of the thermopile were measured with a vacuum photoelectric cell having an S-5 photocathode. The photocell was mounted in a small aluminum case containing a 45-volt hearing-aid battery. The system was then connected with shielded cable to a DC electrometer amplifier (General Radio Type 1230-A). The photoelectric cell was calibrated at each wavelength with the thermopile.

BIOLOGICAL MATERIAL: Husked oat seed (*Avena sativa*, Victory 2020) were soaked one hour in distilled water and imbedded on the surface of 1.0 % distilled water-agar slants in 10×75 mm test tubes. They were grown for 48 hours in a red irradiation cabinet at 10 μw/cm² (625 to 1000 mμ) to inhibit elongation of the first internode and then transferred to a dark cabinet for 17 to 22 hours. Fifteen seedlings were rigorously selected over the safelight for uniform straightness and length of 20 ± 2 mm, and the tubes secured in wooden holders with rubber bands. Each holder contained five tubes for the blue irradiated controls and 10 for the monochromatic irradiation. The seedlings were mounted 13 mm apart at an average distance of 150 cm from the exit slit. The growing room, including the treatment cabinets, was maintained at 25.0 ± 0.5° C and 90 ± 5 % relative humidity.

PROCEDURE: The upper 5 mm of the 10 coleoptiles were irradiated for a constant exposure time of 30 seconds to the monochromatic energy variables. The upper 5 mm of the controls were exposed for one minute to the standard blue source which produced an average curvature of 25.0 ± 1.6°. The holders were irradiated at an angle of 45° to the respective incident beams (fig 1). This procedure introduced an irradiance variation for the seedlings at both ends of about ± 10 % from the axial or mean value. Since the variation was the same in each experiment and the values were averaged, this factor was neglected in the calculations. After irradiation, the holders were rotated an additional 90°, placed in the adjacent cabinet, and shadowgraphed. This procedure made it possible to obtain shadowgraphs of the curvatures at right angles to the coleoptile bending plane without having to rotate the seedlings individually in the holder. Ninety minutes after the first exposure, a second shadowgraph was made on the same film. The angles of curvature were measured from the pair of shadowgraphs with a grid marked to the nearest degree. The curvatures given in figure 2 should be multiplied by cos 45° to obtain actual curvatures. Since this factor does not alter the slopes of the response-log energy curves or the relative values of the action spectra, it was not carried through the calculations.

ANALYSIS OF DATA: The responsivity was determined from 350 to 520 mμ at 10 mμ intervals. In

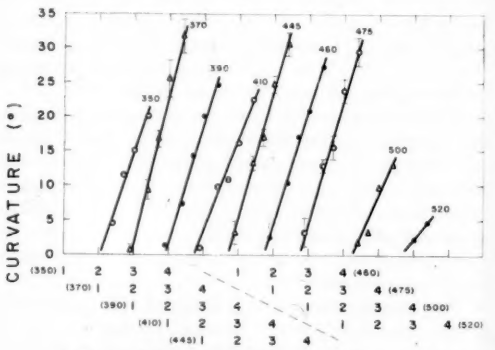

FIG. 2. Response-log energy curves for principal wavelengths. The abscissa is shifted one unit to the right for each wavelength. Probable errors are given for the responses at 370, 445 and 475 mμ.

the vicinity of the peak regions, intermediate 5 mμ determinations were made. The average curvature of 40 to 60 seedlings was measured for four or five incident energies at each wavelength. The curvature, θ, in degrees of angle, was plotted against the logarithm of the incident energy E_i in nano-joules/cm² (nano-joule = 10^{-9} joules/cm²) from 3 to 30°. Regression lines were calculated by the method of least squares to give the best straight line fit, $\theta = m \log E_i + b$. The slope and intercept constants, m and b, are given in table II. The apparent threshold energy, E_0, is defined for each wavelength as the value of E_i when the curve is extrapolated to zero response, $\theta = 0$. The regression lines in figure 2 were drawn for only 9 of the 23 wavelength stations. The logarithmic scale of the abscissa is shifted one unit to the right for each wavelength to avoid superposition.

The action spectrum was plotted as the reciprocal of the moles of incident quanta, n′, required at each wavelength to produce a given response. [Compare with quantum responsivity (10).] The action spectrum was calculated from the incident energy E_i in nano-joules/cm² and the wavelength λ in mμ by the conversion factor:

$$\text{Quantum activity} = \frac{1}{n'} = \frac{120 \times 10^3}{\lambda E_i} \text{ (cm}^2\text{/nano-einstein)} \quad (1)$$

TABLE II

CONSTANTS FOR RESPONSE-LOG ENERGY CURVES

WAVELENGTH Mμ	SLOPE, m DEGREE /LOG′ NJ/CM²)	INTERCEPT, b DEGREE	THRESHOLD ENERGY, E_0 LOG(NJ/CM²)
350	14.9	−30.2	2.03
360	20.5	−40.8	1.99
365	22.0	−43.5	1.98
370	22.7	−44.5	1.96
375	22.2	−43.4	1.95
380	21.4	−41.7	1.95
390	17.2	−33.0	1.92
400	12.9	−23.2	1.80
410	13.2	−23.2	1.76
420	15.2	−26.7	1.76
430	16.1	−28.1	1.74
435	16.4	−28.6	1.74
440	17.5	−29.8	1.70
445	18.3	−31.2	1.70
450	17.7	−30.1	1.70
460	16.4	−28.4	1.73
470	16.7	−28.3	1.69
475	17.5	−30.0	1.71
480	17.2	−29.5	1.71
490	14.4	−28.5	1.98
500	12.0	−27.5	2.29
510	7.3	−17.1	2.34
520	6.2	−16.0	2.58

$\theta = m \log E_i + b$, where

θ = response (degree of curvature)
m = slope
E_i = incident energy
b = ordinate intercept
$E_0 = E_i$ when $\theta = 0$

RESULTS

The action spectra obtained for 0, 5, 10 and 20° responses are given in figure 3. Maxima occur in the visible between 410 to 415, 440 to 445, and 470 to 475 mμ. A near-ultraviolet peak occurs at 370 to 375 mμ and is more evident the larger the response. The variable magnitude indicates some sort of interaction between two pigment systems. Separation cannot easily be made of the activity of the two pigment systems, flavin or carotenoid, based on the presence or absence of a near-ultraviolet peak. It also was observed that the slopes of the response-log energy curves (fig 2) are a function of wavelength and not a constant value as reported previously for phototropism in Avena (6). It is this change in slope with wavelength which is responsible for the variable height of the near-ultraviolet maximum.

DISCUSSION

Phototropic curvature, caused by a unilateral stimulus of radiant energy, is the result of a difference in effect on the two sides of the coleoptile. This difference is produced by the attenuation of the incident energy in its transmittance through the coleoptile. The attenuation is due to three factors: 1) self-screening by the photoreceptor, A_S, 2) neutral scattering, A_N, and 3) foreign screening by inert pigments, A_F. These factors can be grouped as the total absorbance of the coleoptile, A_T, where $A_T = A_S + A_N + A_F$. Absorption then produces a photoproduct, which indirectly controls cell elongation. The total amount of photoproduct depends upon the incident energy, E_i, and the absorption coefficient of the photoreceptor, β. Thus the magnitude of the difference in growth effect on each side (the response) is a function of three factors, the attenuation, the absorption coefficient of the photoreceptor, and the incident energy. This is the case, regardless of the secondary mechanism which actually regulates cell elongation, whether it is auxin redistribution, auxin

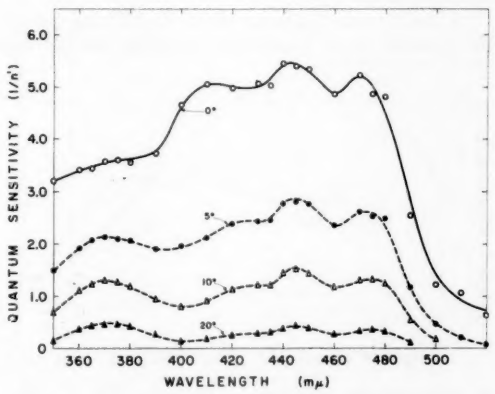

FIG. 3. Action spectra calculated for constant responses 0, 5, 10 and 20°.

inactivation or the destruction of an enzymatic locus necessary for auxin-regulated cell growth.

From the above considerations, a general equation describing the response can be written.

$$\theta = k\, A_T\, (\log E_i + \log \beta) \qquad (2)$$

The response is proportional to the total amount of photoproduct formed and the attenuation of the energy across the tissue.

To obtain the photoreceptor absorption spectrum, the attenuation factor must be excluded. From equation 2, simply set $\theta = 0$ to get

$$\frac{1}{E_i} = \beta \qquad (3)$$

which is the defined action spectrum. Thus the action spectrum obtained for the threshold response will correspond to the photoreceptor absorption spectrum. The action spectra plotted from the energies required for larger responses are a mixture of effects due both to the multiplier A_T and absorption coefficient β. The best procedure, theoretically, would be to measure the response over the range of the first and second positive curvatures and plot log θ instead of θ versus log E_i. Then for each wavelength a curve would be obtained which would be congruent with the curve obtained for every other wavelength. The attenuation factor could be precisely determined from the shift along the ordinate and the action spectrum from the shift along the abscissa. This was attempted with the present data, but it was impossible in practice to separate the two factors. Extending the measurements over the first and second positive curvatures would insure characteristic inflection points for precise comparison.

A plot of the slopes of the response-log energy curves is given in figure 4. The definite near-ultraviolet peak suggests that the curvatures produced in this region were primarily due to an increase in the attenuation. The maxima in the visible are the same as those shown in the action spectra and are presumably due to attenuation by self-screening of the photoreceptor.

A 2nd possible explanation of the 370 mμ peak is the presence of a fluorescent substance having high absorption in the near-ultraviolet. It would absorb energy and then re-emit it in the blue where it could be reabsorbed by the photoreceptor. It would thus not only increase the attenuation, but also increase the concentration of effective photoproduct. In support of this idea, we have observed that coleoptiles irradiated with high intensities of the mercury 365 line, several hundred μw/cm^2, appear to have small, irregularly shaped areas which fluoresce blue. These were observed visually in the section 0.5 to 1.0 mm below the tip with a long focal-length microscope. Attempts have been made to measure the magnitude of this fluorescence and how it is a function of the incident energy. Experimental difficulties of obtaining sufficient sensitivity while at the same time screening out all of the incident blue energy have prevented precise measurements. One of the degradation products of riboflavin, lumichrome, has a near-ultraviolet absorption peak and fluoresces in the blue. Galston and Baker (7) added lumichrome to sections of pea epicotyl for straight growth measurements, but could find no significant effects in the light. However, to obtain a 10° curvature, a difference in length of the two sides of the coleoptile of only 2.0 to 4.0 % is needed. Differences of this order of magnitude would not have been detected in the straight growth type of experiment.

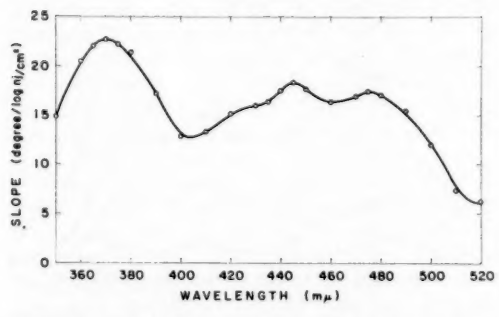

FIG. 4. Slope of response-log energy curves versus wavelength.

The three photoreceptor maxima obtained from the threshold action spectrum in the visible tend to disprove Bünning's (2, 3, 4) hypothesis of a screening mechanism in which a flavin is the photoreceptor. If the flavin were the photoreceptor, the threshold curve would have had a smooth single maximum. Further evidence against the active participation of a flavin is the recent work of Mer (9) in which he could find no evidence which conclusively supports the in vivo photoinactivation of auxin by a flavin.

Recently, Crane and Beinert (5) described an electron transferring flavoprotein (ETF) which has three maxima in the blue at 410, 438 and 460 mμ and a single broad peak about 370 mμ. Two of the blue maxima are apparently due to the protein moeity, since the absorption spectrum of the flavin released from ETF by acid hydrolysis has a characteristic smooth single maximum in the blue. It was tempting at first to consider this as the photoreceptor on the basis of the action spectra for responses larger than 5°. However, the threshold curve again, with the absence of the near-ultraviolet peak, eliminates ETF as the probable phototropic receptor.

SUMMARY

Detailed action spectra of the first positive tip-curvature of Avena have been determined for 10 mμ steps from 350 to 520 mμ. Steps of 5 mμ were determined in the peak regions. From the action spectra it is concluded that the active photoreceptor is a yellow pigment, probably carotenoid in nature, having absorption maxima at 410 to 415, 440 to 445, and 470 to 475 mμ. A flavin photoreceptor appears unlikely, although a peak was observed in the near-ultraviolet

at 370 mμ. Evidence is presented to show that these curvatures may be due to increased attenuation across the coleoptile tissue by an inactive secondary pigment having an absorption maximum at about 370 mμ.

The authors are indebted to Dr. Max Delbrück for his recent theoretical analysis of the phototropic response data presented in this paper.

We wish to thank Dr. Harland Stevens, U.S.D.A. Branch Experiment Station, Aberdeen, Idaho, for supplying the Avena seed.

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EFFECT OF BICARBONATE ON THE ENZYMIC SYNTHESIS OF LONG CHAIN FATTY ACIDS[1,2]

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The metabolic role of carbon dioxide in higher plants is complex. Photosynthetic carboxylation (11) as well as the incorporation of carbon dioxide into oxalacetate (6) and malate (10) have been studied extensively on an enzymatic level in plant materials. In addition carbon dioxide has been implicated in the regulation of several gross responses in higher plants. These effects include the induction of fermentation in aerobic tissues (2), regulation of photoperiodic induction (7), and the control of stomatal opening (3). Recently the inhibition of cytochrome c oxidation-reduction by carbon dioxide has been reported in castor bean mitochondria preparations (1).

We wish to report the requirement of bicarbonate for the synthesis of long chain fatty acids from acetate by a soluble enzyme extract prepared from avocado particle acetone powders. A similar effect has been reported by Wakil and coworkers in their soluble avian system (9).

The components required for the condensation of acetate to long chain fatty acids by the soluble avocado enzyme preparation are listed in table I. By a combination of gas liquid chromatography and reversed phase kerosene paper chromatography the major product of the reaction has been identified as

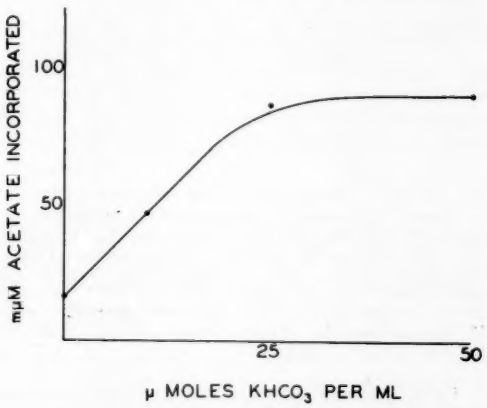

Fig. 1. The effect of bicarbonate concentration on synthesis of long chain fatty acids. Conditions same as for table I, except $KHCO_3$ concentration as indicated.

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[2] Supported in part by a grant from the National Science Foundation.

[3] Predoctoral Fellow of U. S. Public Health Service.

TABLE I

COFACTOR REQUIREMENTS OF THE SOLUBLE AVOCADO ENZYME SYSTEM

COFACTOR OMITTED	MILLIMICROMOLES ACETATE INCORPORATED
None	235
ATP	1
CoA	7
$MnCl_2$	9
$KHCO_3$	15
Olive oil	55
TPN	168
Cysteine	161

The complete reaction mixture contained, in 1 ml: 1 micromole acetate-1-C^{14}; 100 micromoles potassium phosphate, pH 7.5; 5 micromoles ATP; 0.05 micromole CoA; 1 micromole $MnCl_2$; 30 micromoles $KHCO_3$; 0.08 micromole TPN^+; 1 micromole glucose-6-phosphate; 5 micromoles cysteine; 2.5 mg emulsified olive oil; 25 γ glucose-6-phosphate dehydrogenase (prepared by the method of Kornberg (4)); 3 mg soluble protein obtained from avocado particle acetone powders. The reaction mixture was incubated for 60 minutes at 30° C under N_2. The reaction was stopped by addition of an equal volume of saturated $KHSO_4$. The lipid fraction was extracted with $CHCl_3$, evaporated in planchets and counted directly.

free oleic acid; the remaining product is palmitic acid. No short chain intermediates accumulate in the reaction mixtures (8). Of importance is the requirement for TPN^+[4] rather than DPN^+ which participates in the β-oxidation sequence (5), the pronounced requirement for oil emulsions which may be replaced by Tween 40 or Tween 80, and the marked depression of synthesis when bicarbonate is omitted. Figure 1 relates the synthesis of long chain fatty acids as a function of bicarbonate concentration. Since the optimum pH for incorporation of acetate into fatty acids is 7.5, with a marked decrease below and above this value, stimulation of synthesis by bicarbonate cannot be related to pH effects on the enzyme systems.

TABLE II

EFFECT OF BICARBONATE CONCENTRATION ON THE SYNTHESIS OF FAT BY AVOCADO PARTICLES

MICROMOLES BICARBONATE ADDED	PERCENT ACETATE INCORPORATED
0	9
5	17
10	23
20	22
40	23

The reaction mixture contained: 0.1 micromole acetate-1-C^{14}, 200,000 cpm; 140 micromoles TRIS-HCl, pH 7.2; 175 micromoles sucrose; 10 micromoles ATP; 1 micromole $MnSO_4$; 0.07 micromole CoA; 5 micromoles glutathione; 0.15 micromole DPN^+; 0.07 micromole TPN^+; 10 micromoles citrate; particle suspensions (about 10 mg protein) (8). Final volume: 1.8 ml. Incubated for 2 hrs at 32° C under N_2.

[4] The following abbreviations are used: adenosine triphosphate, ATP; coenzyme A, CoA; diphosphopyridine nucleotide, DPN^+; triphosphopyridine nucleotide, TPN^+; glutathione, GSH; 2-amino-2-hydroxymethyl-1,3-propanediol, TRIS.

Table II summarizes the effect of varying bicarbonate concentration on the biosynthesis of fat from acetate by freshly prepared avocado particles. The effect is not as marked; however considerable increase in biosynthesis is noted at higher bicarbonate concentrations. Although variable results have been obtained with avocado tissue slices, at higher concentrations of bicarbonate (20 micromoles) stimulation is frequently noted.

The mechanism of action of this remarkable effect is obscure. The incorporation of labeled bicarbonate into either long chain or volatile fatty acids does not occur. Whatever the nature of the effect it would appear that synthesis of fat in the plant cell is controlled in part by carbon dioxide concentration.

We are indebted to Calavo Growers of California, Escondido, California, for a generous gift of avocados.

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EFFECT OF PETROLEUM ETHER EXTRACTION AND READDITION OF VARIOUS COMPOUNDS ON THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLASTS[1]

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The discovery in this laboratory by Lynch and French (1) that β-carotene partially restores the photochemical activity lost by petroleum ether extraction of chloroplasts suggests that this pigment is an active component of the photosynthetic mechanism. This observation prompted the present inquiry to determine whether β-carotene is specific for such reactivation or whether other fat soluble components in the petroleum ether extract may replace or complement the β-carotene effect. In the course of these studies certain refinements in techniques for isolation of chloroplasts and for measuring photochemical activity were introduced.

Lynch and French (1) found that the extracted material usually retained a substantial fraction of its original activity, and that readdition of the petroleum ether extract restored its photochemical activity to a larger extent than did the addition of pure β-carotene. In the present work, we found it possible to reduce the activity by petroleum ether extraction nearly to zero by changing the method of isolating the chloroplast material. The reactivating effect of a number of substances other than β-carotene was tested. None of these equalled the reactivation effect of adding back the intact petroleum ether extract.

MATERIALS AND METHODS

Chloroplasts were isolated by two procedures from Swiss chard (*Beta vulgaris* L., var. cicla) and from pokeweed (*Phytolacca americana* L.), and were lyophilized. One method, that employed by Lynch and French, is similar to those used by a number of other investigators. Chloroplasts are released from leaves in a Waring blendor with ice-cold 0.5 M sucrose solution in 0.025 M phosphate buffer, pH 7.5. The other isolation technique substitutes 15 % methanol for 0.5 M sucrose, in the same buffer solution. This modification is based on the previous observation in this laboratory (2) that dilute solutions of methanol stabilize the photochemical activity of aqueous suspensions of chloroplast material.

Optimal homogenization of leaf material was obtained with a ratio of 200 g of chard or 150 g of pokeweed to 300 ml of solution. Fresh leaves were weighed after removal of midribs. After homogenizing one minute in the blendor, large leaf debris was removed by forcing the suspension through an ordinary household strainer. This facilitated filtration through closely woven muslin to remove the rest of the debris. The chloroplast material was sedimented at 10,000 × G for 30 minutes. Lyophilization was facilitated by partial removal of the sucrose by resuspending and washing the first plastid sediment in ice-cold distilled water. After this treatment, a thick suspension of the plastids was deposited on the inner wall of the lyophilizing flask by immersing it in a mixture of dry ice and Cellosolve. The washing step was omitted when the methanol-buffer mixture was used. Isolation and subsequent treatment of the chloroplast material was done in a cold room or a cold water bath.

Determination of chlorophyll content of lyophilized chloroplasts, necessary for calculation of photochemical activity in absolute units, was carried out by the method described by H. W. Milner et al (2).

Petroleum ether used for extraction of dry chloroplasts was distilled in glass and two fractions, boiling point 26 to 50 and 50 to 70° C, were collected. Dry chloroplasts, 24 mg, were extracted in a homogenizing tube with a closely fitting Lucite plunger by two grinding treatments at 5° C with 12-ml portions of petroleum ether, applying 12 strokes of the plunger each time. Following each extraction treatment, the chloroplasts were centrifuged for 5 minutes at 1000 × G. The decanted extracts were pooled and retained. The petroleum ether extracted plastids were then resuspended in 12 ml of cold low-boiling petroleum ether. Before photochemical measurements, a 2-ml portion of the well-stirred suspension, containing 4 mg of chloroplasts, was transferred to the homogenizer. The petroleum ether was evaporated at reduced pressure with continuous shaking. The evaporation kept the chloroplasts cold. Either a portion of the original petroleum ether extract, or of a petroleum ether solution of some substance to be tested, could be added to the tube and mixed by homogenization with the chloroplast suspension before evaporating the petroleum ether. After removal of the petroleum ether, the plastids were resuspended for photochemical activity measurements by homogenizing with 10 ml of 15 % methanol in 0.04 M phosphate buffer, pH 6.5.

Chloroplasts lost their activity very rapidly (as much as 50 % in 30 minutes in the cold) when suspended in aqueous phosphate buffer, following the technique of Lynch and French. However, when 15 % methanol was substituted for the aqueous component of the phosphate buffer, the photochemical activity of chloroplasts stayed intact for as long as one hour, even at room temperature.

Photochemical measurements were made with a Beckman DK-2 spectrophotometer, using 4-ml glass-stoppered cells with one window of opal glass, this window being to the rear of the cell compartment. The cells were kindly supplied by Dr. Kazuo Shibata.

[1] Received revised manuscript June 17, 1958.

[2] Visiting Investigator. Address: Kansas State College, Manhattan, Kansas.

A 3.5-ml aliquot of the 10-ml chloroplast suspension was placed in a cell. Its absorbance value at 600 mμ (the absorption maximum of 2,6-dichlorophenolindophenol) was recorded as a line on the paper. Then 0.5 micromole of dye was added (0.5 ml of 0.001 M dye in 0.04 M phosphate buffer, pH 6.5), the cell contents mixed, and the absorbance of the mixture recorded. The optimum dye concentration was predetermined by using screens to find the maximum permissible value which would permit maintaining light saturation. Addition of the dye increased the absorbance of the suspension by approximately 0.5. The Beer-Lambert law was found to hold for this system, so that absorbance values for the dye could be related directly to its concentration.

After recording the absorbance of the chloroplast-dye mixture, the cell was removed from the holder and illuminated at 6000 ft-c for successive 30-second intervals. After each illumination, the photochemical activity produced was recorded in terms of decrease in absorbance. The paper was moved a centimeter to the left after the recording of each point. The suspension was mixed thoroughly before and after each illumination. Although the activity during the first one half minute is of primary concern, since it indicates the initial activity, four more illuminations were usually made in order to verify the trend of the dye reduction.

A typical example of raw graphical data obtained in this way, using freshly isolated and lyophilized chloroplasts from pokeweed, is shown in figure 1. Activity measurements in terms of dye reduced are based on graphical extrapolation of the first 30-second rate to one minute, and use of the following formula for calculations:

$$\text{Activity (micromoles dye reduced per mg chlorophyll per hour)} = \frac{A \times 60 \times B}{C \times D \times E}, \text{ where}$$

A = percentage of the dye reduced by one minute of illumination (extrapolated graphically from first one half minute period).
B = micromoles of dye added before illumination.
C = milliliters of aqueous buffered suspension of chloroplasts.
D = milligrams of chloroplasts per ml suspension.
E = percentage of chlorophyll in chloroplasts.

In the case illustrated by figure 1, measurement of the extrapolated one half minute value showed dye reduction at the rate of 69.2 % per minute. Thus the photochemical activity was $\frac{69.2 \times 60 \times 0.5}{3.5 \times 0.4 \times 9} = 155$ (micromoles dye/mg chlorophyll/hour).

Figure 1 shows that the decrease in dye concentration with time is not linear, but follows a more or less logarithmic form. With prolonged light exposure this downward dye bleaching curve would turn upwards again due to a photooxidation whose rate eventually exceeds that of the photoreduction.

A number of fat-soluble compounds were added in petroleum ether solution to extracted chloroplasts

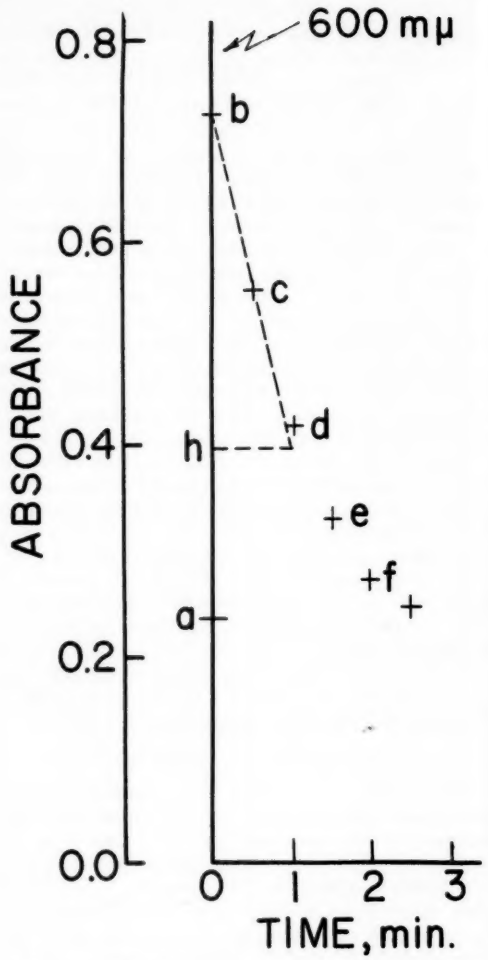

FIG. 1. Typical data obtained with a Beckman DK-2 recording spectrophotometer on photochemical reduction in opal glass cells of 2,6-dichlorophenolindophenol by lyophilized chloroplasts suspended in phosphate buffer (15 % methanol). Absorbance at 600 mμ is recorded after illumination intervals of 30 seconds at 6000 ft-c. Recording paper is moved 1 cm to left after each illumination interval. a—Absorbance of buffered chloroplast system before adding dye. b—Absorbance of buffered chloroplast system after addition of 0.5 micromoles of 2,6-dichlorophenolindophenol, before illumination. c, d, e, f—Absorbance values after successive half-minute illumination periods. h—Residual absorbance of chloroplast-dye system after 1 minute illumination, obtained by extrapolation of the initial half-minute value.

Percentage of dye reduced in first minute $= \frac{(b-h) \times 100}{(b-a)}$.

and the solvent removed in vacuum. β-carotene from four sources was used, one a commercial sample from Mann Research Laboratory and three preparations by Dr. H. H. Strain, including one from chard and one from beet leaves. Solutions of these, containing 0.22 or 0.10 mg per ml, were added to extracted chloroplasts to give various concentrations up to 1.5 mg pigment per mg chloroplasts. Other substances such as lecithin, tocopherol, etc. were dissolved in petroleum ether, 1 mg per ml, and the effect of various amounts of such substances on the reactivation of extracted chloroplasts was studied.

Results

Chlorophyll Content: Two preparations of lyophilized chard chloroplasts had 7.0 and 7.3 % chlorophyll on a dry weight basis. Four pokeweed chloroplast preparations had 9.4, 8.7, 9.4 and 8.4 % chlorophyll respectively. Accordingly, a chlorophyll content of 7.0 % was used to calculate activity of chard chloroplasts and 9.0 % for pokeweed chloroplasts. Extraction by low-boiling petroleum ether removed about 1 % of the chlorophyll content.

Source of Chloroplasts and Effect of Method of Isolation: Some of the preliminary work was done with chloroplasts from Swiss chard, while pokeweed chloroplasts were used for the bulk of the work. The earlier work showed that chloroplasts from both sources behave similarly. Since pokeweed chloroplasts were used in all final phases of the study, only data pertaining to pokeweed chloroplasts are presented in the tables.

Following the development of the techniques described, confirmation was obtained of the results reported by Lynch and French in inactivating chloroplasts by treatments with low-boiling petroleum ether, and reactivating them by adding back the extract. However, reactivation was not observed in several experiments when β-carotene was added at the concentration indicated by Lynch and French to be effective. It was discovered later that β-carotene in approximately 100 times greater concentration than that used by them was effective.

The clear-cut reduction of photochemical activity by extraction with petroleum ether and its restoration with β-carotene as accomplished in the previous experiments were not duplicated with subsequent preparations of either pokeweed or chard chloroplasts. It was finally discovered that chloroplasts prepared with buffered sucrose behaved quite differently in this regard from those isolated with buffered 15 % methanol. Accordingly, experiments were made to ascertain the efficiency of removal of photochemical activity from sucrose-prepared and methanol-prepared plastids, using different petroleum ether fractions and different extraction procedures, and to determine the reactivating effect of β-carotene and other substances. Sucrose- and methanol-prepared chloroplasts frequently showed differences in response to the same additive.

Petroleum Ether Extraction of Sucrose-prepared Chloroplasts: Typical results of many experiments of this type are shown in table I. In marked contrast to results obtained with chloroplasts isolated using methanol (table II), those prepared with sucrose retain a large part of their photochemical activity after extraction with petroleum ether. Material prepared with sucrose fell only from 238 to 102 (table I, lines 1 and 2) after extraction with cold low-boiling petroleum ether. Drastic extraction of sucrose-prepared chloroplasts in a Soxhlet apparatus for 18 hours at room temperature (line 3) did not

Table I

Effect of Petroleum Ether Extraction of Sucrose-prepared Pokeweed Chloroplasts and Their Reactivation by Different Substances

	Treatment	Additive	Photochemical activity *
	Preparation VII		
1	None	None	238
2	Ext. 2× with 26 to 50° C petroleum ether	None	102
3	Ext. 18 hr in Soxhlet with 26 to 50° C petroleum ether	None	25
4	Ext. 2× with 50 to 70° C petroleum ether	None	7
5	Plastids from 3 re-ext. 2× with 50 to 70° C petroleum ether	None	5
6	Plastids from 4	1.0 mg β-carotene	31
	Preparation IX		
7	None	None	180
8	Ext. 3× with ordinary petroleum ether	None	35
9	Plastids from 8	10 ml extract	111
10	Plastids from 8	2.0 mg β-carotene	43
	Preparation X		
11	None	None	184
12	Ext. 3× with ordinary petroleum ether	None	46
13	Plastids from 12	10 ml extract	66
14	Plastids from 12	0.5 mg α-carotene	72
15	Plastids from 12	0.5 mg cryptoxanthin	54

* Micromoles dye reduced per mg chlorophyll per hour.

inactivate them completely. Brief treatment with high-boiling petroleum ether (line 4) did, however, cause a sharp loss in photochemical activity. Treatment with high-boiling petroleum ether also reduced considerably the residual activity of material previously extracted with the low-boiling fraction (line 5). Three extractions with undistilled petroleum ether permitted the retention of activities of 35 and 46 out of an original 180 and 184 (lines 7 and 12).

Reactivation of Sucrose-prepared Chloroplasts: It is significant that the reactivation effect obtained with β-carotene is considerably reduced when isolation is carried out with sucrose, in spite of the fact that these chloroplasts, when treated with petroleum ether, may still be reactivated by the extract. Adding back the extract to the chloroplasts (line 9) produced excellent restoration of activity to 111. Nevertheless, β-carotene (line 10) produced only slight reactivation. Although adding back the total extract produces a response in photochemical activity about on a par with that obtained with methanol-prepared chloroplasts, the β-carotene reactivation effect is small. Besides β-carotene, α-carotene and cryptoxanthin activated sucrose-prepared chloroplasts after their extraction with petroleum ether (lines 14 and 15). Cryptoxanthin had but a small effect.

In order of increasing effect, corn oil, mixed tocopherols, white mineral oil, paraffin, ethyl oleate and lecithin were inhibitory to the activity of extracted sucrose-prepared chloroplasts.

Petroleum Ether Extraction of Methanol-prepared Chloroplasts: Characteristic experiments with chloroplasts isolated with 15% methanol are shown in table II. It is seen that extraction of the lyophilized chloroplasts with only two 12-ml portions

Table II

Effect of Petroleum Ether Extraction of Methanol-prepared Pokeweed Chloroplasts and Their Reactivation by Different Substances

	Treatment	Additive	Photochemical activity *
1	None	None	185
2	Ext. 2× with 26 to 50° C petroleum ether	None	2
3	Plastids from 2	Extract **	61
4	"	1.5 mg β-carotene	37
5	"	0.75 " "	49
6	"	0.44 " "	21
7	"	0.30 " "	13
	Preparation XI—1		
8	None	None	167
9	Ext. 2× with 26 to 50° C petroleum ether	None	1
10	Plastids from 9	0.5 ml extract	44
11	"	1.0 " "	71
12	"	5.0 " "	122
13	"	0.2 mg β-carotene	35
14	"	0.4 " "	32
15	"	1.0 " "	44
16	"	2.0 " "	29
	Preparation XI—2		
17	None	None	167
18	Ext. once with 26 to 50° C petroleum ether	None	37
19	Plastids from 18	0.3 mg cholesterol †	43
20	"	1.0 " "	39
	Preparation XI—3		
21	None	None	167
22	Ext. 2× with 26 to 50° C petroleum ether	None	8
23	Plastids from 22	2 mg β-sitosterol	20
24	"	2 mg cholesterol ††	15
25	"	1 mg cryptoxanthin	41
26	"	1 mg α-carotene	36
27	"	10 ml saturated solution of lutein	49
28	None	None	165
	Preparation VI		
29	Ext. 2× with 26 to 50° C petroleum ether	None	2
30	Plastids from 29	5 mg β-carotene	66
31	"	8 ml extract	83
32	"	5 mg cholesterol †	52

* Micromoles dye reduced per mg chlorophyll per hour.
** Extract from 8 mg chloroplasts added to 4 mg chloroplasts.
† Eastman, old stock.
†† Nutritional Biochemicals, fresh supply.

of low-boiling petroleum ether resulted in virtually complete elimination of the photochemical activity. This value fell from 185 before extraction to 2 afterward, or from 167 to 1 (lines 1, 2 and 8, 9).

REACTIVATION OF METHANOL-PREPARED CHLOROPLASTS: Adding back the extract from 8 mg of plastids to 4 mg of extracted material restored the activity to 61 (line 3). Optimum β-carotene reactivation of this preparation occurred at approximately 1 mg per mg of chloroplasts (lines 4 to 7), a relatively massive addition. The maximum activity regained by adding β-carotene was about one fourth that of the unextracted plastids, and only about four fifths of that produced by adding back the extract. Cryptoxanthin (line 25) showed a definite positive effect on methanol-prepared plastids. Also α-carotene and lutein (lines 26, 27) clearly activated these preparations.

Extracted methanol-prepared chloroplasts were inhibited in increasing order by mixed tocopherols, wheat germ extract, ergosterol, stigmasterol and phytol.

THE ANOMALOUS EFFECT OF CHOLESTEROL: Early in the work it was observed that cholesterol appeared to activate some preparations of extracted chloroplasts and to inhibit others. Later, it became apparent that cholesterol appeared to be effective for the plastids prepared with methanol, but inhibitory for sucrose-prepared ones. Characteristic positive results are shown in table II. Definite reactivation was observed with preparation VI (line 32). However, other methanol-prepared chloroplasts exhibited only small reactivation with cholesterol (lines 19, 24). Two samples of the sterol were used, one from Eastman Kodak Co. that had been on hand for a number of years, the other a fresh lot from Nutritional Biochemicals Corp. No reactivation by cholesterol was comparable to that by β-carotene. It seems probable that the effect of cholesterol may be rationalized either on the basis of its structural similarity to a compound in chloroplasts vital to photochemical activity, or to some impurity in the cholesterol. Since cholesterol is not a normal constituent of chloroplasts, and other sterols from plant sources produced no reactivation, weight is added to the possibility that some contaminant in the cholesterol was responsible.

ABSORBANCE CHANGES IN EXTRACTED CHLOROPLASTS: In a search for changes related to differences in photochemical activity of chloroplasts caused by petroleum ether extraction, absorbance spectra of various chloroplast preparations were examined in the range of 400 to 750 mμ, using the opal glass cells of Shibata. Such spectra are shown in figure 2. Fresh chloroplasts suspended in aqueous buffer show the typical peaks of chlorophyll at 430 to 440 and 670 to 680 mμ, and a strong carotenoid band at 470 to 480 mμ. Methanol-prepared chloroplasts extracted twice with low-boiling petroleum ether, which reduced the photochemical activity from 167 to 1, showed no significant changes in absorbance (except for some reduction of the carotenoid band) which could be related to the drastic change in activity. The petroleum ether extract of this preparation showed strong absorption bands of carotenoids. Chlorophyll bands were absent. On the other hand, when a chloroplast preparation is extracted exhaustively with low-boiling petroleum ether, then is re-extracted with the high-boiling fraction, a significant amount of chlorophyll (red band) appears in the high-boiling extract, with carotenoids virtually absent. The reduction in photochemical activity of chloroplasts extracted with high-boiling petroleum ether (table I) may be associated with this difference in effect of the two petroleum ether fractions.

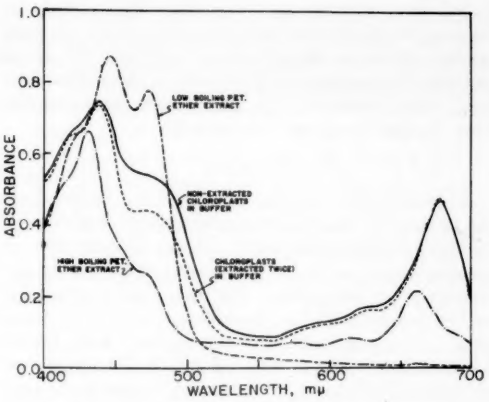

FIG. 2. Typical absorbance spectra in the visible range using opal glass cells, of aqueous suspensions of lyophilized chloroplasts and of petroleum ether extracted chloroplasts, as well as of the petroleum ether extracts of lyophilized chloroplasts.

DISCUSSION

The data presented here clearly confirm the finding by Lynch and French concerning the involvement of β-carotene in the photochemical step of photosynthesis. The results suggest further that this effect is non-specific, since α-carotene, cryptoxanthin, and lutein also produce some reactivation of chloroplasts extracted with petroleum ether. The activation by cholesterol is less clear cut and may be caused by contaminants in the preparations used. It is logical to speculate that some moiety of the carotenoid molecule may be responsible for the activation.

A significant finding was the markedly greater removal of photochemical activity of chloroplasts when methanol rather than sucrose was employed in their isolation. The use of methanol made possible the reduction of photochemical activity by petroleum ether extraction to such low values that reactivation effects by means of carotenoids could be more clearly demonstrated. Little difference in the degree of reactivation was noted between sucrose- and methanol-prepared chloroplasts when the petroleum ether extract was used as the activating agent. It may be con-

cluded that chloroplasts prepared with sucrose are resistant to extraction by petroleum ether of components vital to photochemical activity. It seems probable that sucrose is adsorbed to chloroplast surfaces, thus inhibiting the penetration of petroleum ether during the extraction process.

Summary

Photochemical activity of lyophilized Swiss chard and pokeweed chloroplasts, measured by reduction of 2,6-dichlorophenolindophenol during illumination of aqueous suspensions, can be largely removed by petroleum ether extraction. The extracted plastids are readily reactivated by β-carotene, and to a lesser extent by α-carotene, cryptoxanthin, and lutein. Substitution of 15 % methanol for 0.5 M sucrose during isolation of the chloroplasts facilitated lyophilization and permitted virtually complete removal of activity by brief treatment with low-boiling petroleum ether. Reactivation effects were more clear-cut with methanol-prepared chloroplasts. Buffered 15 % methanol solution also markedly inhibits the rapid loss of photochemical activity found when chloroplasts are suspended in a buffered sucrose-containing medium. Absorbance spectra of aqueous suspensions of petroleum ether extracted chloroplasts in the visible range, as well as of various petroleum ether extracts, do not explain the loss of activity of chloroplasts by extraction, except for the involvement of β-carotene.

In every case of reactivation by β-carotene the effect was modest in comparison with the activity of unextracted plastids, as well as with the activation produced by adding back even small amounts of the petroleum ether extract. For this reason it is inferred that substances other than β-carotene also contribute to the reactivation. Of the substances tested, α-carotene, lutein, cryptoxanthin, and sometimes cholesterol had an activating effect.

Fat-soluble substances that inhibited the residual activity of extracted chloroplasts were corn oil, white mineral oil, mixed tocopherols, paraffin, wheat germ extract, ergosterol, stigmasterol, phytol, ethyl oleate, and lecithin.

The results suggest that the reactivation may be due in part to a molecular fragment common to carotenoids and to some non-carotenoid fat-soluble compounds as well.

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Note added in proof: Bishop has recently reported similar experiments on inactivation of freeze-dried spinach chloroplasts by petroleum ether extraction. Inconsistent results were obtained on readdition of purified β-carotene so many other additions were tried. Vitamin K_3 (Menadione) and Vitamin K_5 were as efficient as the extract itself in restoring activity, under conditions where purified samples of β-carotene were ineffective. Addition of β-carotene with Menadione did, however, prevent a photoxidation which occurred with Menadione alone. This note is a condensation of: Bishop, N. I. The role of certain petroleum ether-soluble compounds in the Hill reaction. Plant Physiol. 33 Suppl.: *xxvii*. 1958.

EFFECTS OF TEMPERATURE ON GROWTH AND DRY MATTER ACCUMULATION OF PEAS[1]

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Growth of peas (*Pisum sativum*) has been studied extensively in the Earhart Plant Research Laboratory, primarily in terms of stem elongation (4). In the present paper some experiments are presented in which dry matter production in peas has been followed.

Unica pea plants (*Pisum sativum*), started from unvernalized seeds, were grown in a 1 : 1 mixture of vermiculite and gravel and were watered three times a week with nutrient solution. After 20 days at 20° C day-temperature and 14° C night, 165 uniform plants with 4 to 5 leaves were selected and transplanted to individual plastic cups (February 17, 1956). Twenty-one plants were immediately harvested while the remaining 144 were distributed over four treatments to allow four successive harvests of 9 plants each.

Treatments were as follows: 8 hours artificial light (800 to 900 ft-c) and 16 hours darkness at the following temperatures: 10/10, 10/20, 20/10, and 20/20 (photo- and nycto-temperatures respectively). Harvests were carried out at 8-day intervals; various measurements and/or dry weight determinations were made on harvested and remaining plants. Leaf areas were measured from shadowgrams of the leaves.

Results and Discussion

Results of the first experiment are shown in table I insofar as the dry weight is concerned. All data were analyzed statistically. This showed that while

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[2] Research Fellow at Earhart Plant Research Laboratory, from December 1, 1955 to April 30, 1956, while on sabbatical leave from the Hebrew University. He gratefully acknowledges facilities provided by the Earhart Plant Research Laboratory and its Staff.

the coefficient of variability for dry weight of the seeds is 14.0 %, for that of the initial harvest, it is 11.1 % for leaves, 10.3 % for shoots, 14.6 % for roots, and for the partially depleted cotyledons it is 46.1 %. Therefore the seed reserves of these peas are irregularly used to produce leaves and stems of very uniform weight, more uniform than the original seed material (cf. Went (3)).

Figure 1 presents the stem length of the peas as a function of age, under the four different temperature treatments. When compared with the data on dry weight it shows that growth rate and dry matter production do not parallel each other.

Total dry weight at 10/10 is highest (in every harvest), attaining its maximum advantage over other treatments in the 3rd harvest (fig 2). This is partly due to the persistence of cotyledons. Dry weight of treatment 10/20, which shows a pronounced chlorosis of leaves, is by far the lowest. For stems, however, the rate of elongation in smallest for 10/10 and increases through 10/20 and 20/10 up to 20/20.

Even though the phototemperature lasted for 8 hours only, and the nyctotemperature for 16 hours, figure 1 shows that the temperature during the light period has a considerably stronger effect on stem elongation than the nyctotemperature.

The 10/20 compared with the 20/10 treatment produced a larger number of nodes (12.7 vs. 11.9); internodes, however, were shorter (1.63 cm vs. 1.91 cm). Both of the above differences were significant at the .01 level.

Root growth of peas is optimal at about 10° C (4). This is seen in this experiment both in the dry weight of roots (table I) and the top/root ratio of their dry weight, which is lowest for the 10/10 treatment. If we plot dry weight of roots against degree-hours per daily cycle (fig 3), it becomes evident that root growth is negatively correlated with degree-hours, the slope of the regression lines increasing in successive harvests (−0.06, −0.10, −0.14, −0.19).

Relative growth rates and net assimilation rates, both on a leaf weight and a leaf area basis (1), were calculated. Two interesting features of these relationships are the initial negative values for treatments at a 20° C night-temperature and the late decline of values for treatments at a 10° C day-temperature (fig 4).

Some preliminary experiments on translocations of assimilates at night were carried out with the twin leaflet method using both detached and undetached leaves. One leaflet was collected at the end of one day from plants growing in natural light and 20°/14°

TABLE I

DRY WEIGHT DETERMINATIONS OF DIFFERENT PARTS OF PEA PLANTS

TREATMENT DAY/NIGHT TEMPERATURE °C	TOTAL DRY WEIGHT OF PLANTS MG	DRY WEIGHT OF LEAVES MG	DRY WEIGHT OF STEMS MG	DRY WEIGHT OF COTS MG	DRY WEIGHT OF ROOTS MG	DRY WEIGHT OF UNIT STEM LENGTH MG/CM
			January 28: dry seeds *			
../..	241.0	...	...	...	...	...
			Initial harvest, February 17 *			
20/14	155.2	39.6	30.7	36.7	48.2	...
			Harvest I, February 25			
10/10	179.3	59.0	36.4	25.5	58.4	3.17
20/10	170.0	60.0	33.1	25.4	51.5	2.71
10/20	141.5	50.6	29.0	22.7	39.3	2.43
20/20	147.2	56.2	30.3	14.6	46.1	2.38
			Harvest II, March 4			
10/10	217.0	68.6	43.5	25.3	79.6	3.29
20/10	185.2	72.3	44.9	3.4	64.6	2.91
10/20	175.3	72.7	42.3	...	60.2	2.72
20/20	171.2	74.2	43.1	...	53.8	2.49
			Harvest III, March 12			
10/10	255.8	95.2	49.7	19.7	91.2	3.42
20/10	214.2	89.3	50.1	...	74.8	2.65
10/20	184.3	77.4	45.8	...	61.1	2.52
20/20	182.0	69.4	53.8	...	58.8	2.34
			Harvest IV, March 20			
10/10	274.3	110.2	58.8	0.9	104.4	3.38
20/10	244.6	103.7	62.8	...	78.1	2.72
10/20	176.8	65.6	52.1	...	59.1	2.51
20/20	220.3	93.1	66.7	...	60.5	2.33

* Figures for January 28 and February 17 are averages of 21 seeds or plants; for later dates, of 9 plants.

C, and the other from plants or leaves (with petiole immersed in water) left for about 15 hours in darkness at 10° or 20° C. Total weight loss was calculated from undetached leaves and loss through respiration from detached leaves.

The results, although somewhat erratic (probably because of varying amounts of reserves present in leaves at the end of each day) seem to indicate a greater or at least not lesser translocation at 10° than at 20° C (see table II). This confirms the conclusions reached in the case of tomatoes by Went (2) and by Went and Hull (5).

In conclusion it seems that although a temperature of 10° C for peas is not optimal for elongation and unfolding of new leaves (the lowest number of nodes is found at 10/10), the largest root systems develop at this temperature. Their growth can occur optimally since translocation seems to be highest at lower temperatures. With increasing age we find higher weights for both stem length and leaf area units (both significantly higher at the .01 level in 10/10 than in any other treatment) possibly as a consequence of photosynthate storage. Plants growing at 10/10 also begin to branch (IV harvest) while no branching is found at 20/20.

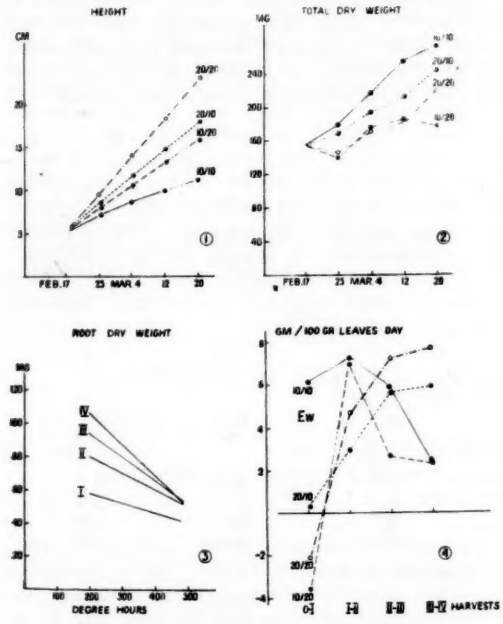

FIG. 1. Height of plants, cm (successive measurements of plants sampled on the last harvest; 10/10, 10/20, etc., day and night temperatures in °C).

FIG. 2. Total dry weight, mg, of plants sampled at different dates.

FIG. 3. Regression lines of dry weight of roots on degree-hours per daily cycle, at different harvests (I, II, III, IV).

FIG. 4. Net assimilation rates (Ew) in grams per 100 grams dry weight of leaves per day, during the successive harvest intervals.

TABLE II

TRANSLOCATION AND RESPIRATION LOSSES DURING 15 HOURS OF DARKNESS *

DATE	°C	TOTAL WEIGHT LOSS	RESPIRATORY LOSS	TRANSLOCATION LOSS
January 21	20	8.95	6.32	2.63
" "	10	5.86	2.23	3.63
February 2	20	15.33	4.46	10.87
" "	10	10.82	0.80	10.02
March 8	20	13.95	6.20	7.75
" "	10	11.75	2.00	9.75

* In percentage of dry weight initially present; averages of 4 replicates of 20 to 30 leaves each.

SUMMARY

Dry matter accumulation and elongation of Unica pea plants growing at two levels of day/night temperatures (10°/10° C, 10/20, 20/10, 20/20) over a period of one month were investigated.

Elongation is greatest at high day-temperatures (20/20, 20/10) even if this means a lower total of degree-hours per daily cycle.

Conversely, dry matter accumulation is highest at 10/10, followed by 20/10 and 20/20. The lowest is at 10/20 treatment, where leaf chlorosis is pronounced.

Growth of roots is best at 10/10 and therefore also top/root ratio is lowest, because of depressed growth of tops and unhampered or even increased translocation to roots; some data corroborating this view are presented.

Curves of relative growth rate and net assimilation rate show initial negative values for treatments with 20° C night temperature and late decline for treatments with 10° C day temperature.

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PRODUCTION, TRANSLOCATION AND ACCUMULATION OF ALKALOIDS IN TOBACCO SCIONS GRAFTED TO TOMATO ROOTSTOCKS[1,2]

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The remarkably low alkaloid content of whole tobacco shoots grown as scions on rootstocks of low-alkaloid species has been recorded by many investigators (cf. Dawson (5), James (8), and Mothes (10) for reviews). Although the data of Tso and Jeffrey (14) appeared to be in contradiction, the disagreement has since been resolved (see below). Solt (13) demonstrated that low alkaloid content in this material could not be the result of an interaction between substantial rates of synthesis and destruction. Consequently, the earlier explanation (4) involving a low intrinsic rate of alkaloid production in the shoot was accepted.

Solt (13) also described the distribution of alkaloid in grafted shoots preparatory to an analysis of the marked disparity in alkaloid synthesizing capacities of root and shoot. The present paper describes the changes in such distribution brought about by removal of the apical bud (topping), defoliation, top-grafting and approach-grafting, respectively, of scions of two varieties of tobacco grafted to tomato roots. Deductions are drawn regarding the probable locus of synthesis, currents of transport and major sites of accumulation of alkaloid in the system tobacco scion–tomato rootstock.

MATERIALS AND METHODS

GENERAL MANAGEMENT OF THE CULTURES: Plants of *Nicotiana tabacum* L., vars. Connecticut Shade No. 49 and Turkish (Samsun), cigar and cigarette types, respectively, were grown in the greenhouse during the winter and spring months. Cleft-grafts were prepared between these tobaccos and the Marglobe variety of tomato, *Lycopersicum esculentum* Mill. When the 1st graft unions were healed, all plants were transferred to clay pots of 12-inch diameter containing a rich potting soil. Surface applications of a complete fertilizer mixture were made periodically to maintain vigorous growth. Axillary shoots were removed from the plants as rapidly as they appeared.

SCHEDULE OF EXPERIMENTAL TREATMENTS: Approximately three weeks after grafting, when the growth rate of the tobacco scions was restored to nearly normal, the plants, both grafted and non-grafted, were divided into lots according to subsequent treatment as follows.

1) One lot of nongrafted plants was allowed to grow as above without further treatment and was designated "nongrafted controls."

2) One lot of grafted plants of each variety of tobacco was designated as "grafted controls" and allowed to grow without further treatment. This group is referred to as once-grafted plants in the text that follows.

3) When the tobacco scions of the grafted plants had attained a stem length of ca. 250 mm, a 3rd lot was selected from which the tops were removed, leaving ca. 6 leaves on the Connecticut 49 scions and 9 leaves on the Turkish scions. These plants were still further subdivided into four groups, viz. (a) One group was harvested immediately for analysis. (b) A 2nd group was top-grafted with Marglobe tomato. This graft combination was designated tomato-tobacco-tomato, the first-named representing the rootstock species and the last the uppermost scion species. (c) A 3rd group was top-grafted with Marglobe tomato, and 20 days later the tobacco components were defoliated. (d) The 4th group was top-grafted with the same tobacco variety and was hence designated tomato-tobacco-tobacco. These four groups were also termed short-stem, twice-grafted plants.

4) When the tobacco scions of the remaining once-grafted plants had attained a stem length of ca. 800 mm, a 3rd lot was removed and subdivided according to further treatment as follows. (a) One group was harvested immediately for analysis. (b) A 2nd group was topped by removal of the terminal bud. This group was designated long-stem, once-grafted and topped. (c) A 3rd group was top-grafted with Marglobe tomato scions. This group was termed long-stem, twice-grafted. The Connecticut 49 scions retained 11 leaves and the Turkish scions 20 leaves, respectively.

5) Approach grafts were made between a few of the remaining once-grafted plants and intact plants of tomato. When the tobacco scions were about 300 mm in length, they were grafted at a point about 180 mm above the soil surface to the stem of a tomato plant of equal height. The point of union between tomato and tobacco shoots was thus about one half the vertical distance between their respective bases and apical buds. The graft union was prepared by removing tangentially from each graft partner, a piece of stem about 2 cm long and one half the thickness of the stem. The cut surfaces were tied together until healing occurred. Leaves were present above and below the approach-graft union, and both graft partners retained their root systems. However, 15 days after grafting the apical bud of the tobacco component was removed (topped).

SAMPLING AND CHEMICAL METHODS: From three to five plants were collected at each sampling, and most experimental populations were sampled for assay purposes at two or three stages of development. The

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[2] This paper is based on work peformed under Atomic Energy Contract No. AT(30-1)-1778.

individual plants were divided into their various parts, viz.: tomato rootstocks, tobacco scion stem, tobacco scion leaves, tomato scion leaves and stem, etc. In some cases the tobacco and tomato scion stems were cut into segments about 150 mm in length. Graft unions were removed by making two transverse cuts such that the resulting stem segment contained all of the wound healing tissue associated with both stock and scion. These graft unions were analyzed separately. Except where specifically stated the alkaloid and dry weight figures are excluded from yield calculations.

The plant material was dried in a forced-draft, hot-air oven at ca. 70° C. In order to facilitate drying, the thick stems and midribs were cut into slices. The dried samples were ground in a Wiley mill using a 40-mesh screen.

Total steam-volatile pyridine alkaloid content was estimated by the methods of Willits et al (15) as modified in this laboratory (6). The specific extinction coefficient of nicotine was employed in the calculations, and alkaloid content is given as nicotine.

Nicotinic acid labeled with tritium in the 2- position was prepared by treating 2-bromo-3-picoline with lithium butyl and subsequently with tritium oxide by methods to be described elsewhere. The method of tritium assay was that of Wilzbach, Kaplan and Brown (17) as modified by Christman (2) for use with glass proportional counting tubes.

Interpretation of the Data: Direct comparisons between analytical data obtained from the different treatments are somewhat complicated by the time requirements for graft wound healing (ca. three weeks in most cases). Therefore, an attempt was made to sample the different populations when comparable stages of development (height and reproduction) were reached without regard to chronological age. Attention should be called to the fact that twice-grafted plants were, in effect, topped plants also for the periods of time involved in graft wound healing.

Practical limitations of space and difficulty of producing uniform material are responsible for the small size of each sample. The magnitude of many of the observed differences is sufficiently great, however, to compensate for this loss of experimental efficiency.

Although, as indicated above, two varieties of tobacco were employed in these experiments, the results were qualitatively the same in both cases. For this reason, no attempt is made in the sections that follow to present complete data for both species.

Results

Effects of Treatments on Alkaloid Yields: In agreement with earlier findings, the mean alkaloid content of once-grafted tobacco shoots was lower than that of ungrafted shoots by two orders of magnitude (cf. Dawson (4); Solt (13)). The data are contained in table I.

Unexpected, however, was the virtual restoration of the total alkaloid normal to the variety simply by removing the terminal bud (topping) of the tobacco scion (table I). That the effect was real is indicated by the results of twice-grafting in which a tomato apical bud replaced the tobacco bud. The intermediate alkaloid content in the latter case may have resulted in part from the increased rate of alkaloid production during the three weeks following the 2nd grafting when the tobacco scion was in essentially a topped condition.

Table I

Total Nicotine and Dry Weight of Various Graft Combinations Between Connecticut 49 (long-stem) and Tomato

Treatment	Mean dry weight g	Mean nicotine mg
Nongrafted	49.2 ± 5.9 *	177.9 ± 64.0 *
Tomato-tobacco	43.9 ± 7.3	0.6 ± 0.4
Tomato-tobacco (topped)	84.7 ± 5.3	145.9 ± 32.5
Tomato-tobacco-tomato	71.4 ± 15.7	47.4 ± 9.1

* Standard deviation.

That the production and accumulation of alkaloid in twice-grafted plants were continuing processes is shown by the data of tables II, III and IV. The alkaloid content of the tobacco scion increased following the 2nd grafting but tended to become stabilized in later periods. The tomato scion continued, however, to accumulate alkaloid throughout. The similarity of this trend in accumulation patterns to that described for once-grafted plants in an earlier paper should be noted (13). In a modification of this experimental approach, the tomato scion of a twice-grafted, (tomato-tobacco-tomato) short-stem plant was cut back close to the uppermost graft union. An axillary bud was allowed to develop a new tomato shoot. After 46 days of growth 19 mg of alkaloid were found in the regenerated tomato shoot. The tobacco scion in this case was variety Connecticut 49.

Defoliation of the tobacco scion component in short-stem, twice-grafted plants not only failed to reduce the rate of alkaloid accumulation but, in fact, appeared to increase it (table II). This increase was, however, paralleled by an increase in dry weight of the tobacco stem (see below).

When long-stem, twice-grafted plants were assayed for alkaloid, the results were only quantitatively different. The tomato scions did not grow as vigorously in these graft combinations as in the case of the short-stem, twice-grafted plants. Nevertheless, the total alkaloid content was substantially greater in these long-stem, twice-grafted shoots (cf. data in tables I and II) than it was in the short-stem, twice-grafted shoots.

Effects of Treatments on Alkaloid Distribution: The distribution of alkaloid within twice-grafted plants (tomato-tobacco-tomato) is given in tables II, III, IV and V. Several points may be emphasized. First, the total alkaloid content of the tomato scion component is always less than that of

the tobacco component regardless of treatment. Second, the graft unions, regardless of position, and the lowermost stem segment (150 mm) of the tobacco scion accumulated relatively high amounts of alkaloid both absolutely and relatively. Third, the tomato stock was always entirely free of alkaloids so far as the sensitivity of the assay method permits such a conclusion. Fourth, the concentrations of alkaloid in the leaves of tobacco and tomato scions were approximately the same within a given treatment. Fifth, the patterns of alkaloid distribution in stem segments of tomato and tobacco scions were greatly different (cf. tables IV and V); in tobacco there was a strong diminution from base toward the top, while in tomato there was no pronounced gradient. Sixth (table V), the distribution of alkaloid in the upper tobacco scions of the combination tomato-tobacco-tobacco was similar to that of the tobacco scion component of the combination tomato-tobacco-tomato as given in table IV and unlike that of the tomato scion in the latter combination.

VARIETAL EFFECTS ON ALKALOID YIELD AND DISTRIBUTION: Table III contains data which may be used with the data of table II to afford a comparison of tobacco varietal effects in the twice-grafted plants. Qualitatively, the patterns of alkaloid distribution are the same; the principal difference is the result of the higher alkaloid yielding potential of the Turkish variety (cf. Solt (13)).

ALKALOID DISTRIBUTION IN TOBACCO SCIONS AND TOMATO PLANTS JOINED BY AN APPROACH-GRAFT UNION: The distribution of alkaloid among various parts of an approach-grafted complex is given in table VI. The tobacco component of this grafted complex contained relatively large amounts of alkaloid with the highest concentration per unit dry weight occurring below the approach-graft union (but above the cleft-graft union with tomato rootstock). The amounts of

TABLE II

DISTRIBUTION OF NICOTINE AND DRY WEIGHT WITHIN SHOOTS OF TWICE-GRAFTED CONNECTICUT 49 TOBACCO (SHORT-STEM)

TREATMENT	DAYS AFTER SECOND GRAFTING	TISSUE	MEAN DRY WEIGHT G	MEAN NICOTINE MG
Tomato-tobacco	0	Tobacco leaves	7.6 ± 1.3 *	0.5 ± 0.1
		Tobacco stem	1.8 ± 0.7	1.0 ± 0.2
		Total	9.4 ± 1.9	1.6 ± 0.1
Tomato-tobacco-tomato	28	Tobacco leaves	10.7 ± 2.7	4.0 ± 1.7
		Tobacco stem	2.8 ± 1.1	9.6 ± 3.8
		Tomato leaves + stem	16.2 ± 2.3	4.6 ± 2.4
		Graft union I **	1.8 ± 0.4	4.0 ± 2.3
		Graft union II †	1.4 ± 0.4	2.1 ± 1.5
		Total ††	29.6 ± 3.8	18.4 ± 6.9
Tomato-tobacco-tomato	69	Tobacco leaves	9.8 ± 3.4	3.6 ± 2.7
		Tobacco stem	3.6 ± 1.1	10.0 ± 1.5
		Tomato leaves	26.8 ± 5.3	9.9 ± 3.9
		Tomato stem	14.7 ± 1.1	0.8 ± 0.2
		Graft union I **	2.0 ± 0.4	3.1 ± 0.7
		Graft union II †	1.6 ± 0.2	1.5 ± 0.5
		Total ††	54.8 ± 3.3	24.1 ± 5.1
Tomato-tobacco-tobacco	43	Bottom leaves	21.3 ± 2.2	10.6 ± 2.9
		Bottom stem	11.2 ± 2.2	37.8 ± 4.3
		Top leaves	11.4 ± 5.3	6.6 ± 2.9
		Top stem	15.8 ± 6.5	3.5 ± 1.5
		Total	59.7 ± 16.4	57.8 ± 10.4
Tomato-tobacco-tomato (tobacco leaves removed)	43	Tobacco leaves ‡	13.2 ± 0.4	1.4 ± 0.1
		Tobacco stem	4.1 ± 0.8	12.1 ± 4.5
		Tomato leaves	17.6 ± 3.5	7.9 ± 0.8
		Tomato stem	4.1 ± 1.1	0.3 ± 0.1
		Total ‡‡	25.8 ± 5.3	20.4 ± 5.4
Tomato-tobacco-tomato (tobacco leaves removed)	76	Tobacco leaves ‡	15.2 ± 2.9	1.4 ± 0.5
		Tobacco stem	7.0 ± 1.0	25.5 ± 0.8
		Tomato leaves	47.9 ± 7.2	20.1 ± 1.5
		Tomato stem	20.8 ± 2.0	1.7 ± 0.5
		Total ‡‡	75.6 ± 8.2	47.3 ± 1.9

* Standard deviation.
** Union between tomato rootstock and tobacco scion.
† Union between tobacco and tomato scions.
†† Graft union data not included.
‡ Leaves at time of removal, 20 days after 2nd grafting.
‡‡ Tobacco leaf data not included.

TABLE III

DISTRIBUTION OF NICOTINE AND DRY WEIGHT WITHIN SHOOTS OF SHORT-STEM, TWICE-GRAFTED TURKISH TOBACCO (TOMATO-TOBACCO-TOMATO)

DAYS AFTER SECOND GRAFTING	TISSUE	MEAN DRY WEIGHT G	MEAN NICOTINE MG
36	Tobacco leaves	18.9 ± 0.7 *	28.8 ± 6.4 *
	Tobacco stem	5.2 ± 2.5	36.9 ± 13.0
	Tomato leaves	10.2 ± 4.3	11.4 ± 3.2
	Tomato stem	3.3 ± 0.3	1.3 ± 0.7
	Total	38.0 ± 4.1	73.4 ± 22.0
58	Tobacco leaves	19.1 ± 3.8	47.0 ± 13.8
	Tobacco stem	7.3 ± 3.1	43.8 ± 16.6
	Tomato leaves	16.7 ± 4.6	31.3 ± 8.2
	Tomato stem	11.9 ± 1.9	2.2 ± 0.2
	Total	55.1 ± 3.5	124.3 ± 23.5
82	Tobacco leaves	22.0 ± 0.3	37.9 ± 24.2
	Tobacco stem	7.9 ± 3.2	59.0 ± 12.3
	Tomato leaves	43.2 ± 11.0	68.6 ± 24.1
	Tomato stem	26.5 ± 10.5	5.1 ± 1.4
	Total	101.8 ± 23.9	171.7 ± 31.6

* Standard deviation.

alkaloid in the tomato shoot joined to tobacco by the approach-graft were substantially lower and were distributed much the same as in the tomato scions of the twice-grafted combination, tomato-tobacco-tomato. The outstanding feature of such distribution, however, was the occurrence of some alkaloid in the tomato component below the approach-graft union. Similar results were obtained when Connecticut 49 was used as the tobacco component.

FEEDING A LABELLED PRECURSOR OF NICOTINE TO TOMATO SCIONS: To determine whether or not the alkaloid found in the tomato scion of twice-grafted plants was formed in that scion from precursors translocated from the tobacco segment, the following experiment was performed. Five such tomato scions from the short-stem grafts, tomato-tobacco-tomato combination (cf. table II for expected nicotine content), were excised and cultured with their cut stems (2) in an ice-cold solution of 2-tritium-nicotinic acid (6). Fifty mg of the acid were used. Its specific activity was 246,000 dpm/mg. The tobacco scion component was Connecticut 49 variety. Although all of the aqueous solution containing the nicotinic acid was taken up by these excised shoots in the 1st day of culture, water was added to permit the culture period to extend to 6 days. Nicotine dipicrate was obtained from the steam distillate of the tomato tissues. The yield was 103 mg. Tritium assay by Dr. D. R. Christman of Brookhaven National Laboratory showed that the nicotine so obtained was not at all radioactive (0.0 dpm/mg) within the limits of sensitivity of the procedure.

TABLE IV

COMPARISON OF NICOTINE AND DRY WEIGHT OF THE TOBACCO STEM OF TWO CONNECTICUT 49 (LONG-STEM) PLANTS TWICE GRAFTED WITH TOMATO

STEM SEGMENT	TOTAL DRY WEIGHT G		TOTAL NICOTINE MG	
	INITIAL	39 DAYS	INITIAL	39 DAYS
Graft union I	...	4.4	...	11.29
1	2.9	11.2	1.86	35.47
2	2.3	9.4	.05	8.11
3	2.0	8.0	.03	2.56
4	1.8	6.8	.03	1.85
5	1.4	6.1	.00	1.18
Graft union II	...	1.9	...	1.55

One stem was assayed at the time of the 2nd grafting; the other after 39 days of growth. Each stem was divided into segments 150 mm long.

TABLE V

DISTRIBUTION OF NICOTINE IN THE STEM OF THE TOP SCION OF PLANTS INVOLVING DOUBLE GRAFTS BETWEEN CONNECTICUT 49 (SHORT-STEM) AND TOMATO

TREATMENT	MG NICOTINE PER STEM SEGMENT OF TOP SCION				
	GRAFT UNION	1 (BASAL)	2	3	4
Tomato-tobacco-tomato	2.78	.10	.15	.12	.06
Tomato-tobacco-tobacco	4.55	1.89	.50	.44	.32

Stems were divided into sections 150 mm long.

DISCUSSION

LOCUS OF ALKALOID SYNTHESIS: With the use of radioactive nicotinic acid as precursor, it was shown in an earlier paper (13) that the excised tobacco shoot can make small amounts of nicotine. The data of the earlier paper and those described herein point strongly to the tobacco stem, including the graft union, as the locus of much of the alkaloid synthesis that occurred in these grafted plants. The following points sup-

TABLE VI

DISTRIBUTION OF DRY WEIGHT AND NICOTINE WITHIN THE PARTS OF AN APPROACH-GRAFT BETWEEN TOMATO AND ONCE-GRAFTED (ON TOMATO) TURKISH TOBACCO

	TISSUE	DRY WEIGHT G	NICOTINE MG
Tobacco half	Leaves above approach-graft union	20.3	17.5
	Stem above approach-graft union	6.2	18.2
	Leaves below approach-graft union	5.7	0.5
	Stem below approach-graft union	5.7	33.7
Tomato half	Leaves above approach-graft union	24.1	6.3
	Stem above approach-graft union	10.3	0.6
	Stem and leaves below approach-graft union	5.0	0.4

port this conclusion. First, yields of alkaloid obtained from grafted plants that had also been stripped of tobacco leaves (defoliated) were no less than those from nondefoliated once-grafted plants (table II). Second, the sites of greatest alkaloid accumulation in all grafted plants were the graft unions and the tobacco stem segments immediately above the graft unions (table IV). Third, the presence of strong gradients of alkaloid accumulation in the stems of the tobacco scions as well as the absence of such gradients in the terminal tomato scions of the twice-grafted combinations are consistent with this interpretation (see below).

That the graft union with its greater cross-sectional dimensions was not the sole site of alkaloid synthesis is indicated by the following facts. First, the synthesis of radioactive nicotine in excised tobacco scions noted in the first paragraph above was accomplished in the complete absence of graft union tissue (the latter had been removed prior to the experiment). Second, as indicated above, alkaloid distribution in a tobacco stem above the graft union with tomato rootstock was quite different from that in a tomato stem above the graft union with a tobacco scion as in the twice-grafted plants (tables IV and V). If the graft union were the sole site of synthesis it would appear that identical distribution patterns should have occurred. Third, the axial gradient of alkaloid distribution in a tobacco scion superior to a graft union with tomato in the combination tomato-tobacco-tomato is the same as that in both tobacco scions of the combination tomato-tobacco-tobacco (table V). From these facts it follows that the occurrence of such an axial gradient is a characteristic of tobacco, and not of tomato, scions and that, consequently, the high concentration of alkaloid located in the lowermost stem segment of such scions is a result principally of synthesis in situ and not of translocation from the graft union.

The alkaloid found in the tomato scion component of the twice-grafted plants could not have originated in the tomato scion. The lack of radioactivity in the nicotine isolated from tomato shoots fed tritiated nicotinic acid is evidence against synthesis by these tomato shoots. If alkaloid synthesis had occurred in the excised tomato scion, a specific activity of 60 dpm/mg might reasonably have been expected. Such expectation is based on two reasonable assumptions: 1) the rates of alkaloid synthesis in excised shoots of tomato and tobacco should be generally proportional to the mean alkaloid concentrations in intact plants of the two species; and 2) the rates of incorporation of nicotinic acid into nicotine should be proportional to the amount of the acid supplied (unpublished data).

Despite the fact that no substantial decrease in alkaloid production occurred in these experiments when the tobacco leaves of twice-grafted plants were removed, unequivocal evidence is lacking that leaves fail to produce alkaloid at some stage in their development (cf. 9, 13, 14).

PATTERNS OF TRANSLOCATION: There was an accumulation of alkaloid in the terminal tomato scion of all twice-grafted plants of the type tomato-tobacco-tomato. Since it has been shown that tomato shoots do not produce pyridine alkaloids at rates sufficient to account for the concentrations observed, even when supplied with nicotinic acid, it is clear that the amounts reported herein must have arisen entirely from translocation from the tobacco component.

The distribution of alkaloid within the tomato scion component provides further evidence of the validity of this conclusion. Thus, the alkaloid content of the leaves of the tomato scion was higher than that of the tomato stems. Further, as related above, the vertical distribution of alkaloid in the tomato scion stem was relatively uniform throughout. The grafted tobacco stem showed opposite relationships; the stem contained more alkaloid than the leaves and the distribution varied with the distance from the basal graft union. It is noteworthy that in the intact tobacco plant, where the great bulk of the alkaloid of the shoot is brought up from the roots, the distributional pattern in the shoot is similar to that of the tomato scion in the present grafting experiments.

The evidence thus fits the following hypothesis. Nicotine is produced in the stem of the tobacco scion component. It is translocated upward through the xylem (4) principally to the leaves. The 2nd graft union (between tobacco and tomato scions) appears to offer no barrier to upward transport, and thus the alkaloid is deposited principally in the leaves of both scions. Similarities in alkaloid concentration between tobacco leaves (0.34 ± 0.14 mg per g dry weight) and tomato leaves (0.36 ± 0.13 mg per g dry weight) of the plants described in table II (treatment 3) may be a reflection of this postulated freedom of transport.

Judging by the absence of nicotine in the tomato stocks of the graft combinations described above, downward transport of the alkaloid across a graft union of the cleft type apparently never occurs. Absence in this case is related to the sensitivity of the assay method employed. By special methods (13), it is possible to demonstrate the presence of nicotine in the tomato variety used in our grafting experiments whether or not they have been grafted with tobacco plants, but the amounts involved are several orders of magnitude below those described herein. The validity of the above statement is therefore not impaired.

Some downward transport of alkaloid was demonstrated in the tomato component of the approach graft described above. The contradiction in these observations may be removed if it be assumed that a graft union imposes a barrier against downward but not against upward transport of alkaloid. Thus, in case of the cleft graft, no alkaloid movement could occur from tobacco scion to tomato rootstock owing to the fact that the graft union involved the entire cross-sectional area of the stem. In the case of the approach graft, however, nicotine could flow upward

from the tobacco stem, across the graft union, and into the tomato stem superior to the graft union. Earlier data have shown a tendency toward redistribution of alkaloid throughout the stem cylinder above the graft union in such cases (4). In the present experiment, alkaloid so redistributed could then descend through the tomato stem in that sector where there occurred no graft union to bar the way.

With respect to the notion of a barrier to downward translocation, Rogers and Beakbone (12) have correlated decreases in sieve tube diameter and number at the graft union with decreased downward flow of organic materials. It is thus tempting to conclude that alkaloids may also be transported downward in the phloem. However, the barrier to downward alkaloid movement in this case appears to be complete, while the barrier to downward flow of foodstuffs must be only partial judging by the fact that growth and development of the tomato rootstock is normal. The outward manifestation of a lowered rate of downward transport of foodstuffs in our grafted plants is the swelling which always occurs at the graft union. A puzzling aspect of this situation is, however, the fact that much of the observed stem swelling of the tobacco scion is visibly associated with an increased production of vascular tissue, at and near the graft union.

If the downward movement of tobacco alkaloids can occur in tomato shoots it seems very likely that it can also occur in tobacco shoots, although the amounts involved may never be large. Wilson has reached similar conclusions in the case of the alkaloids of *Atropa belladonna* (16).

Patterns of Accumulation: Patterns of alkaloid accumulation in the once-grafted tobacco shoot have been described in detail in earlier sections of this paper and by Solt (13). Special mention should be made of the approximation of an alkaloid content normal for ungrafted shoots of the same variety by once-grafted tobacco shoots which have been topped.

Although it has been known for a long time that the amounts of alkaloid produced and accumulated by a given species or variety are responsive to changes in environment and to cultural manipulation, the extent of the increase brought about by topping of the once-grafted tobacco scion goes far beyond the usually observed order of magnitude. We have been unable to locate comparable observations in the literature. However, the recent paper of Tso and Jeffrey (14) reports alkaloid contents for once-grafted tobacco shoots (tomato rootstock) which are far higher than figures obtained by us or by other workers (10). Private communication with Dr. Jeffrey disclosed that their plants were indeed topped. Hence the discrepancy is removed.

The underlying reasons for the increased alkaloid production due to topping provide interesting subject matter for speculation. If alkaloid synthesis can occur in the stem as earlier concluded, then it must take place at the expense of photosynthate from the leaves and of assimilated (or unassimilated) forms of nitrogen brought up from the roots. The situation immediately above the graft union between tobacco scion and tomato stock is very probably represented by the following. When the apical bud is removed, translocation of photosynthate to the bud and its utilization in new tissue construction are also shut off. The root system represents an alternative primary destination for translocated photosynthate, but there is a partial barrier to such translocation at the first graft union (12). Consequently, there tends to be piling up of such materials at and just above the graft union. The upward flow of nitrogenous compounds from the root system is not hindered by the graft union. Upon passing the boundary between tomato and tobacco tissues at the graft union, these components of the ascending transpiration stream encounter cells which are already heavily loaded with carbohydrate. The result is a greatly increased rate of synthesis of nicotine and possibly of other nitrogenous compounds in the stem tissues within and immediately above the graft union.

Obviously, further work is necessary to ascertain the extent to which gains in alkaloid content of intact tobacco shoots that have been topped are due to a similar interaction within stem or root of the components of the translocation streams. In the past (1, 11) we have inclined to the view that topping merely diverted photosynthate from apical bud and inflorescence development to increases in root growth and synthetic activity without necessarily increasing the alkaloid producing capacity of the histological or anatomical unit. However this may be, it is now clear that the tobacco shoot is potentially capable of synthesizing quantities of alkaloid entirely comparable with those which in earlier experiments have been obtained from the tobacco root system. In the case of the intact tobacco plant, it is also clear that estimates of the net contribution of the shoot to its own alkaloid content, which have been based upon the alkaloid content of once-grafted but untopped tobacco scions, low as they are, may already be too high owing to the effect upon alkaloid synthesis in the scion stems of the barrier to translocation created by the graft union. On the other hand, differences between tobacco and tomato in the composition of the ascending root sap with respect to amounts and kinds of nitrogenous compounds may also affect the rate of synthesis in the tobacco stem. At present, there are no data to assess either the direction or the order of magnitude of such influence.

Summary

Alkaloid yields and distribution patterns have been investigated in the system tobacco scion–tomato rootstock. Consistent with earlier findings, yields were greatly lowered as a result of grafting with tomato rootstocks.

Removal of the apical bud (topping) of the tobacco scion led to a virtual restoration of alkaloid yields normal for the variety. Substituting a tomato

bud for the apical bud of the tobacco scion gave intermediate yields.

Defoliation of the tobacco scion did not reduce alkaloid yields.

Most of the alkaloid of the tobacco scion was located in the graft union and in the stem immediately above the graft union. Such localization was not found in tomato scions grafted upon tobacco scions.

Alkaloid was found in tomato stems and leaves below an approach-graft union with a topped tobacco scion grown on a tomato rootstock.

Labelled (with tritium) nicotinic acid was not converted to nicotine in excised tomato scions that had grown atop tobacco scions.

It is concluded that the principal site of alkaloid synthesis in the system tobacco scion–tomato rootstock is the graft union and the tobacco stem immediately above it. Some of this alkaloid is translocated upward to be deposited in leaf and stem higher on the plant.

Evidence from approach-grafting experiments is interpreted to mean that alkaloid can be translocated downward but that the graft union presents a barrier to such translocation. The possible nature of this barrier is discussed.

It is indicated that the high alkaloid yielding capacity of the graft union and of the adjacent stem tissues is probably atypical. Thus, the direct contribution of the shoot to its own alkaloid content in intact tobacco plants is probably over-estimated when calculations are based upon the alkaloid yields of grafted plants.

Tritium-labeled nicotinic acid was prepared by Dr. Amedeo D'Adamo.

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AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Northeastern Section:* From Buffalo to Orono to Newark, Del., and from many nearer institutions, 131 plant physiologists answered the call to the first Annual Meeting of the Northeastern Section of the American Society of Plant Physiologists, held in Connecticut Hall, Yale University, on May 2nd and 3rd, 1958, with Chairman A. W. Galston presiding.

Following most gracious welcoming addresses by Yale's Provost Emeritus, Edgar S. Furniss, and Department of Botany Chairman, Oswald Tippo, 11 research papers were presented. A social hour and dinner next preceded a discussion of aspects of potentially unlimited growth by Dr. Robert Brown, University of Oxford.

The Saturday morning session consisted of 14 research papers presented by visiting investigators as well as 12 read by title by members of the Yale group. At the noon business meeting officers for 1959 were elected: Dr. T. T. Kozlowski, Chairman; Dr. C. S. Yocum, Vice-chairman; and Dr. H. J. Dyer, Secretary-Treasurer. Drs. Stuart Dunn, Byron E. Janes, and Bruce B. Stowe were appointed to the Executive Committee to represent respectively the New Hampshire, Connecticut, and Massachusetts membership. Grateful appreciation was unanimously voted to Yale University, Chairman Galston, and the Local Committee for their warm hospitality and able labors in behalf of their guests.

Adjournment was followed by a tour of the Department of Botany's enviable facilities in the Josiah Willard Gibbs Laboratory. The tour was conducted by members of the Local Committee and featured discussion and demonstration of current research and equipment.—HUBERT J. DYER, *Secretary-Treasurer.*

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Midwest Section:* The fourth Annual Meeting of the Midwest Section was held at the University of Wisconsin on June 23 and 24, 1958. Approximately 100 members attended the sessions which consisted of research reports and symposia. An experiment was tried in the presentation of the former in that the papers were arranged in advance under 5 headings including, "Membrane Permeability and Translocation," "Plant Growth Substances," "Plant Respiration and Carbohydrate Metabolism," "Nitrogen Metabolism" and "Miscellaneous Papers." Contributed papers were limited to 5 minutes and discussions were reserved for the end of the session or after a series of papers on closely related subjects had been presented. Although it cannot be claimed that these "Report-Question-Retort" sessions were an unqualified success, modifications of this procedure may help to alleviate some of the difficulties due to the multiplication of concurrent sessions.

Symposia on "Dormancy and Spore Germination in Lower Organisms" and on "Acid Metabolism in Plants" also were presented. The annual dinner, presided over by the chairman, Dr. Sam Aronoff, featured a talk by Prof. David Baerreis, Chairman of the Departments of Sociology and Anthropology at the University of Wisconsin, on the subject "Exploring Man's Prehistory." Visits to the Forest Products Laboratory and to the new addition to the Department of Botany, as well as boating on Lake Mendota, rounded out the program.

Officers elected for 1959 were, Dr. Harry Beevers, Chairman, and Dr. Lawrence Bogorad, Vice-chairman. Next year the Midwest Section will meet at Purdue University.

The membership is indebted to Dr. Eldon Newcombe, chairman of the Home Committee, Dr. Robert Burris, chairman of the Program Committee, and to the University of Wisconsin for contributing to the success of these meetings.—ALFRED S. SUSSMAN, *Secretary-Treasurer.*

ENVIRONMENTAL CONTROL OF PLANT GROWTH—Frits W. Went. Ronald Press Co., New York. 373 pages. 1957. $8.50.

Students of plant physiology will find this new book by Dr. Frits Went an extraordinary exposition of the various phases of physiology in which the author is expert. It is extraordinary in that the entire book is sprinkled through with bold and exciting ideas and provocative experiments. As an exposition, it is really a one-man affair, representing chiefly the ideas and personal philosophy of the principal author and frankly making no serious effort to integrate these ideas and points of view with the information reported previously by other authors. It is concerned with various phases of environmental influences upon plant growth and development ranging from photosynthetic ones to subtle ecological factors involved in the control of phenological and geographical features of plant performances.

The first part of the book is concerned with the construction and operation of the Earhart Plant Research Laboratory. In this part more attention is given to a description of the characteristics of this particular laboratory than to the qualities of controlled growth chambers which might be desirable. While the reader can glean much information which will be helpful in the construction and design of new controlled growth chambers, he may find more useful information on this subject in another recent book edited by J. P. Hudson, "Control of the Plant Environment" (Academic Press Inc., New York, and Butterworth's Scientific Publications, London, 1957). Many of the features incorporated into the design of the Earhart Laboratory will be basic to the design of such chambers for years to come, and this description will facilitate the general understanding of the complex machine that it is.

The second part of the book deals with responses of individual species to climatic factors. Many items of information are included which had not been published previously, and it is worthwhile having the

various results for each species brought together in this manner. Especially interesting are the sections on potatoes, peas and strawberries.

The third section, titled General Discussion, consists of an exposition of Dr. Went's ideas concerning the role of environmental factors—principally light and temperature—in growth and development generally. This reviewer found the sections on the analysis of growth, plant climatology and ecology (Chapters 16, 17 and 18) most interesting and offering the most in stimulating ideas.

Five of the chapters have been supplied by other authors, but these take a minor position in the book in that they are more concerned with details and for the most part lack the exciting quality of Dr. Went's style.

The book is generously supplied with illustrations, though the labelling on the graphs is often obscure and requires patient study by the reader before the graph becomes meaningful.

The main feature of the book is its generous supply of provocative ideas. These are ideas of a man with an amazingly detailed knowledge of how plants grow, and while many of the ideas are quite speculative, the reader cannot help finding them stimulating. Some examples of items which appealed to this reviewer are the preference for an inverted thermoperiodic cycle by Saintpaulia (p. 147), the graphic concepts of temperature controls of phenology (p. 228), the analysis of climatic controls of growth rates (p. 232) and the observation that sugar applications can stimulate photosynthesis (p. 278). Some of the points of view expressed were not convincing, such as the proposal of there being diffusion processes which limit growth in medium temperature ranges (p. 215), or the acceptance of stimulations of germination by adding charcoal to the germinating medium as establishing that inhibitors are involved in seed dormancy (p. 264). I was disappointed that the author did not discuss criticisms which had been leveled at some of his ideas in the past. For example, his presentation of the evidence for translocation of sugars having a Q_{10} less than unity is essentially unchanged from the 1949 version although there have been a number of serious criticisms of the concept since then.

The criticisms I have offered are relatively minor. This book will certainly serve a constructive and worthwhile purpose in plant physiology.—A. Carl Leopold, *Purdue University, Lafayette, Indiana.*

Daniel Trembly MacDougal
1865–1958

In these days of narrow specialization in all sciences it is of considerable interest to contemplate the life and activity of a plant physiologist of wide interests and diverse activities. With the death of Dr. D. T. MacDougal, at the age of 92 years, at Pacific Grove, California, on February 22, 1958, another of the few remaining "grand old men" of botany has departed. During his activities as a scientist and investigator of several phases of plant life, MacDougal's name was closely associated, for almost a third of a century, with the establishment of research in plant ecology and physiology in the arid southwestern states by the Carnegie Institution of Washington. In 1903 he helped to organize the Desert Botanical Laboratory near Tucson, Arizona, and soon thereafter became its director. Recognizing the need for a wider scope in laboratory work in plant physiology, he helped to establish and also directed the Coastal Laboratory at Carmel, California, where investigators could extend their studies with plants in a different environment, and work in a more pleasant climate during summer holidays. This laboratory continued in operation until World War II.

While perusing the long list of publications by Dr. MacDougal, one notes the marked influence on his mind (as that of many other biologists during the turn of the century) of the rediscovery of Mendel's classical contribution and establishment of the so-called Mutation Theory by De Vries. This is testified by his many papers on variations, mutations and heredity. MacDougal was editor of De Vries "Species and Varieties." This interest and "first love," though appearing in his publications for several years (Science and Amer. Nat., 1908; Plant World 1909; Bot. Gaz. and Plant World 1911; Ann. Mo. Bot. Garden, 1915) eventually was submerged by his interest in environmental and physiological responses of plants, specifically under desert conditions, concurrent with the establishment of the research laboratories in Arizona and California. Soon Dr. MacDougal was recognized as one of the leading authorities on desert ecology, cycles in plant life due to weather and on physiological effects of heat, light, soil moisture, and other environmental factors on plant development. He was intensely interested in studies of growth of trees with special references to their hydrostatic-pneumatic systems. The MacDougal Dendrograph, an instrument for automatic recording of volume changes in tree trunks, was an outgrowth of these studies.

Daniel Trembly MacDougal was born near Liberty, Indiana, on March 16, 1865. He grew up on a farm which was homesteaded by his grandparents who came from Scotland. In 1890, he received the B.S. degree in science from DePauw University and, in 1891, the Masters degree from Purdue. This was followed by studies in Leipzig and Tübingen and subsequent visits to several botanical laboratories in England, Germany, and Holland, 1895–96. The Leipzig studies on "the curvature of roots" was the basis upon which Purdue University granted the young non-resident scientist the Ph.D. degree in 1897.

Dr. MacDougal's appreciation of the arid regions of the West very likely stems from his botanical explorations in Arizona and Idaho in 1891–92 while he was an Agent of the U. S. Dept. of Agriculture, and his interest in physiology from his studies abroad and when he was an Instructor and Assistant Professor in Plant Physiology at the Univ. of Minnesota, 1893–

1899. During 1899–1905 he was Director of Laboratories of the New York Botanical Garden, where undoubtedly he gained considerable administrative experience. From 1905, to his retirement in 1933, he was a leading member and in charge of botanical research of the Carnegie Institution of Washington, a position which he filled with distinction.

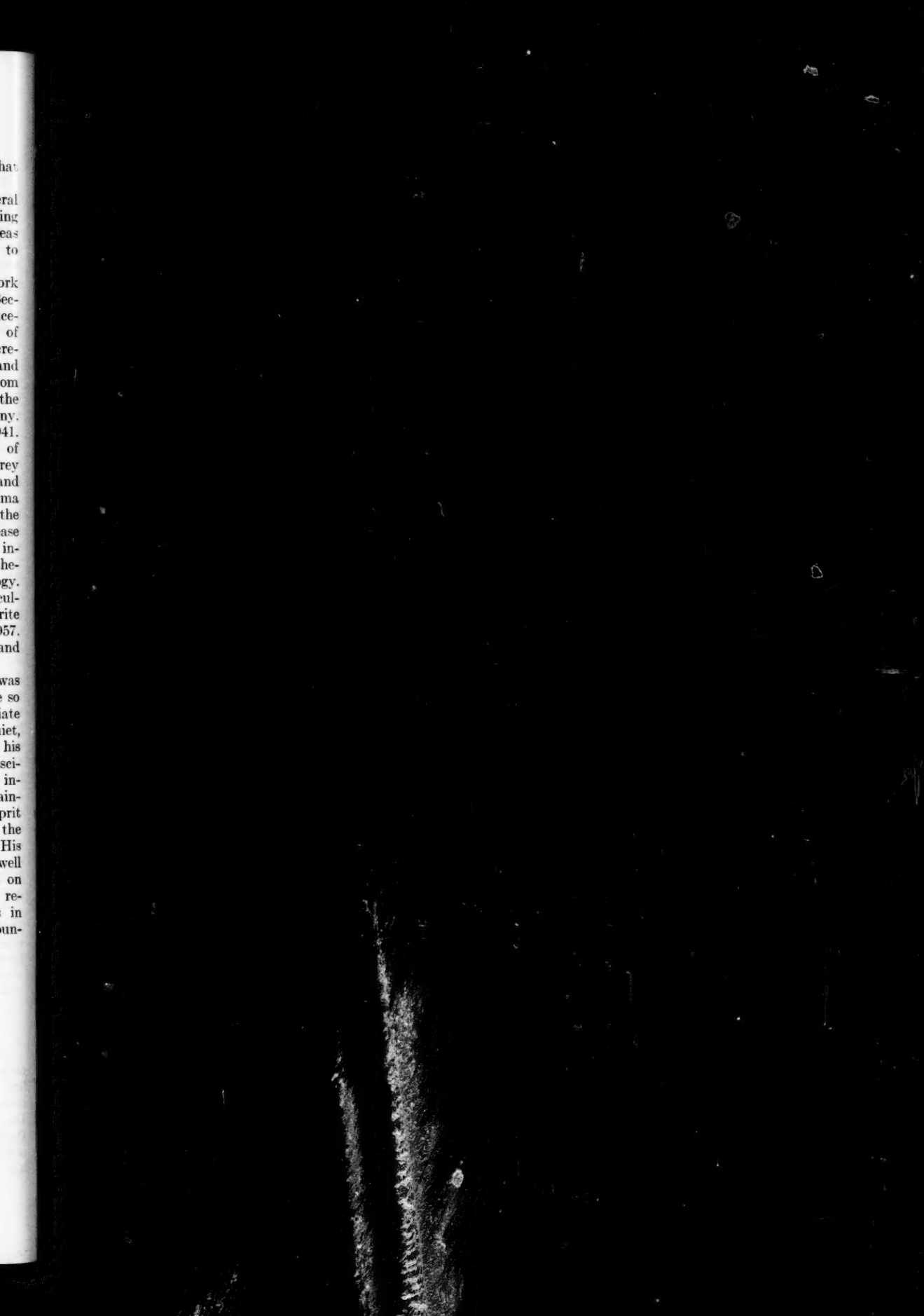
Many honors were conferred on Dr. MacDougal. He was a life member of the Botanical Society of America, the Amer. Soc. of Plant Physiologists, Torrey Bot. Club and the A.A.A.S., of which he was the Gen. Secretary, 1920–25. He was also a member of Hollandsche Maatschappe d. Wetenschappen, Societe Nationale D'Acclimatation de France; hon. member Bot. Soc. of Edinburgh and corr. mem. Czechoslovak Bot. Soc. The Universities of DePauw and Arizona conferred on MacDougal the honorary LL.D. degrees. In 1956 he was given the only Certificate of Distinguished Service ever awarded by the New York Botanical Garden. Dr. MacDougal served as honorary president of the International Botanical Congresses in Stockholm, 1950, and Paris, 1954. He is the author or co-author of about 140 scientific papers and several books and monographs.—A. E. MURNEEK.

SAM FARLOW TRELEASE
1892–1958

Sam Farlow Trelease, Torrey Professor of Botany at Columbia University since 1937 and Executive Officer of the Department of Botany since 1930, died in New York City, February 1, 1958.

Professor Trelease was born in St. Louis, Missouri, July 3, 1892, the son of William and Julia Maria (Johnson) Trelease. He received his A.B. degree from Washington University in 1914, and entered Johns Hopkins University for graduate training in plant physiology. There he worked in the laboratory of Dr. Burton E. Livingston and assisted in courses in Plant Physiology. He obtained his doctorate degree in 1917 and was appointed Assistant Professor at the University of the Philippines where he served from 1917 to 1920. Following this tenure, he returned to Johns Hopkins as an instructor in plant physiology until 1923. In 1923 he joined the faculty at the University of Louisville as Assistant Professor and remained there until 1925. He became Associate Professor of Botany at Columbia University in 1925 and served continuously on the Staff of that institution until his death.

Dr. Trelease's speciality was in the field of mineral nutrition. He has numerous research papers dealing with selenium and spent summers in selenium areas of the Far West where he had an opportunity to study selenium toxicity under field conditions.

Dr. Trelease found time to contribute to the work of many scientific societies. He was Assistant Secretary for the American Association for the Advancement of Science from 1921 to 1923 and Secretary of the Council from 1921 to 1930. He served as Secretary of the Botanical Society of America in 1932 and as Editor of the American Journal of Botany from 1933 to 1939. During this period he developed the present format of the American Journal of Botany. He was elected Vice President of the Society in 1941. He was also a member of the American Society of Plant Physiologists, Phytopathological Society, Torrey Botanical Club, Societé de Botanique de France, and was a member of the honorary fraternities, Gamma Alpha and Sigma Xi. He served on the Board of the New York Botanical Garden since 1928. Dr. Trelease contributed many papers on botanical subjects including mineral requirements of plants, photosynthesis, selenium toxicity, and physiological pathology. He was author of "Laboratory Exercises in Agricultural Botany" published in 1919 and "How to Write Scientific and Technical Papers" published in 1957. A monograph on "Selenium" by S. F. Trelease and O. A. Beath was published in 1949.

Professor Trelease was a great teacher and was highly respected by his students. Those who were so fortunate to study under him learned to appreciate his precise approach to research problems. His quiet, shy manner covered a very warm personality and his graduate students respected him not only for his scientific direction and inspiration, but also for the interest that he took in the social aspects of their training. He promoted scientific fellowship and an esprit de corps that comes from a mutual interest in the scientific accomplishments of fellow students. His greatest contribution to American Botany may well be through the unrecorded influence of the man on his many former students who are carrying on research and education in the Botanical Sciences in laboratories and class rooms here and in other countries.—F. P. CULLINAN.